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Identification and characterization of oligomers of ampicilloic acids impurity in ampicillin sodium active pharmaceutical ingredient in stability studies

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ABSTRACT

An impurity of Ampicillin sodium formed during a gradient reverse phase high performance liquid chromatography (HPLC) analysis of stress stability samples of the drug substance Ampicillin sodium, and the level of this impurity was found at up to 0.9%. This impurity was identified by LC-MS and characterized by (1H NMR, LC/MS/MS, elemental analysis). Based on the spectral data, the impurity was named as, 3-[[(2R,3S)-[3-methoxy-3-N-[2-(thiophen-2-yl)acetamido]]-4-oxoazetidin-2-ylthio]-2-(carbamoyloxy)methyl]]-acrylic acids. The structure of this impurity was also established, prepared by isolation and co-injected into HPLC to confirm the retention time. Structural elucidation further confirms the impurity.

Key words: Ampicillin sodium, Impurities, Isolation,Characterization, LC-MS, NMR, IR

1. INTRODUCTION

β-Lactam antibiotics are the most widely used class of antimicrobial agents. These materials comprise several classes of compounds, among which penicillins and cephalosporins are the most important. These classes contain bulky side chains attached to the 6-aminopenicillanic acid (6-APA) or 7-aminocephalosporinic acid nuclei, respectively[1-3]. The penicillins are of wide usage for their antimicrobial activity against both gram-positive and gram-negative organisms [4,5]. However, the pencillins have limited stability, especially in organic solvents, and produce different degradation products [6,7].

Ampicillin is a semi-synthetic derivative of penicillin, active as a broad-spectrum antibiotic. Inhibits bacterial cell wall synthesis by binding to one or more of the penicillin binding proteins (PBPs); which in turn inhibit the final transpeptidation step of peptidoglycan synthesis in bacterial cell walls, thus inhibiting cell wall biosynthesis. Bacteria eventually lyse due to ongoing activity of cell wall autolytic enzymes (autolysins and murein hydrolases) while cell wall assembly is arrested.

During the analysis of Ampicillin sodium market sample by HPLC-UV method, i.e., similar to European Pharmacopoeia method for Ampicillin sodium API, one impurity was detected at about 0.9%. Hence, the present work was initiated to investigate the nature and origin of the impurity and to characterize it by NMR and mass.

2. EXPERIMENTAL

2.1. Chemicals and reagents

The reagents used for analysis i.e., ammonium acetate (Merck grade), acetonitrile (HPLC grade) procured from Merck (India). High purity water was prepared using Millipore Milli-Q Plus water purification system (Millipore, Milford, MA, USA). Ampicillin sodium samples were procured from market.

2.2. Equipment

Chromatographic separation was performed on a HPLC system with Waters 2695 alliance separations module with Waters alliance 2998 detector and Empower Millienium-2 Software for instrument control and data acquisition. A Shimadzu LC-8A Preparative Liquid Chromatograph equipped with SPD-10AVP, UV-Vis detector [Shimadzu Corporation, Analytical Instruments Division,Kyoto, Japan] was used for semipreparative LC conditions.

2.3. Chromatographic Conditions

The method was developed using Hypersil BDS C 8 250 mm long 4.6 mm i.d., and 5µ particle diameter column (Waters, Milforde, USA) with mobile phase containing a gradient mixture of solvent A (0.01 M ammonium acetate with pH 5.0) and solvent B (Acetonitrile). The gradient program (T/%B) was set as 0.01/2, 20/10, 40/15, 50/25, 60/50, 62/2 and 75/2. The flow rate of the mobile phase was set at 1.0 mL/min. The column temperature was maintained at 25° C and the eluted compounds were monitored at the wavelength of 254 nm. The sample injection volume was 20 µl.

2.4. Semi-Preparative LC Conditions

The separation and isolation of the degradation products were carried out on a semipreparative Hypersil BDS C8 (250 mm long_21.2 mm i.d.) preparative column packed with 8 µ particle size LC column using mobile phase containing solvent A (0.01 M ammonium acetate) and B (Acetonitrile) at a flow rate of 25.0 mL/min. The gradient program (Time (min)/%B) was set 0.01/0, 50/5, 80/10, 100/50, 101/0 and 110/0 and the detector was maintained at 215nm. The column temperature was maintained at 25°C.

2.5. Mass Spectroscopy (HRMS) and NMR Conditions

The HRMS analysis has been carried out by using a Waters Xevo QTOF mass spectrometer. The sample was subjected to LCMS/ ESI QTOF (Waters Xevo QTOF). The 1H and 13 C Nuclear magnetic resonance (NMR) spectra were recorded in DMSO-d6 at 300 MHz and 75MHz, respectively, using Varian Unity INOVA 500 MHz spectrometer (Bruker Biospin, Germany). The chemical shift values were reported on δ scale in ppm with respect to TMS (0.00ppm) and DMSO-d6 (δ 39.5 ppm) as internal standard, respectively.

2.6. Preparation of sample solution

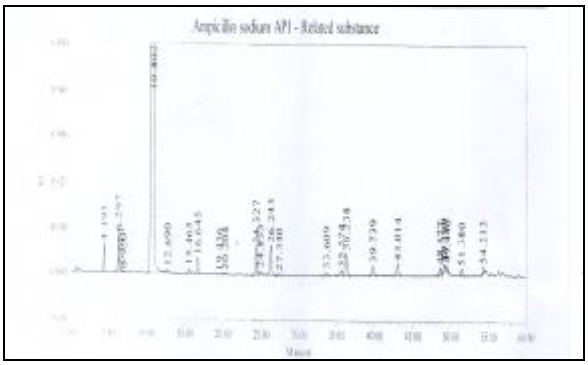
A test preparation of 1000µg/mL of Ampicillin sodium sample was prepared by dissolving appropriate amount in diluents. (Solvent A and B in the ratio of 50% v/v). The LOD and LOQ values of trimer of ampicilloic acids are determined based on the % RSD data, analyzing the diluted sample solutions and injected each solution six times into the HPLC.

3. RESULTS AND DISCUSSION

3.1. Detection of impurities

A critical evaluation of chromatographic data revealed that in few methods, impurities were either merged with each other, or with the main peak. Peak broadening and elution of impurities was also major concern about the specificity of the method. All the methods employed have almost similar chromatographic conditions in isocratic mode. Sample of Ampicillin sodium was analyzed using the gradient high performance liquid chromatography method described in materials and methods section for quantitative chromatography.

Fig-1: Impurity spiked chromatogram of ampicillin sodium



3.2. Enrichment of impurities by semi-preparative HPLC

An impurity with relative retention time (RRT) 2.88 with respect to ampicillin sodium was observed and the level of this impurity was found to be 0.9% (area normalization method) (Fig 2 and 3). The impurity was subjected to semi-preparative isolation conditions as described in instrumentation Section for quantitative chromatography. All the fractions were combined concentrated to 10 mL and was used for LC\MS and NMR analysis. The isolated impurity was co-injected with sample containing impurity and was found in the sample

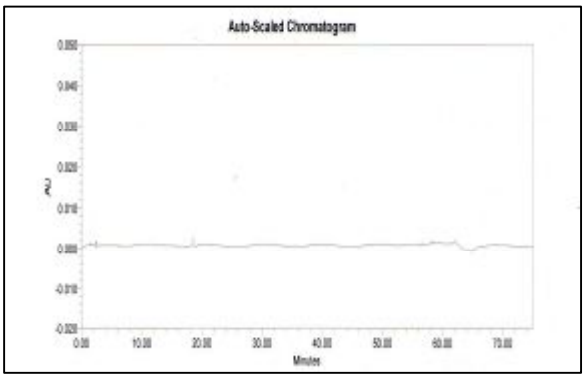
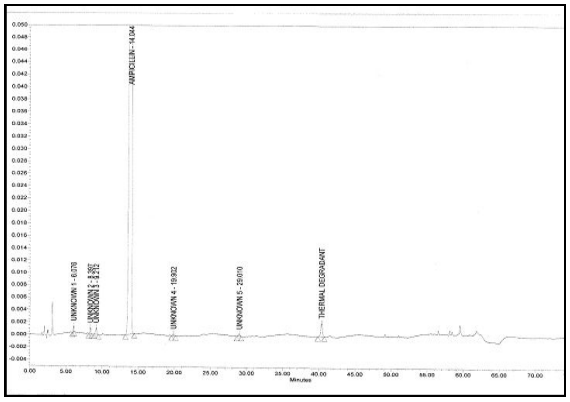


Fig -2: Blank chromatogram of the Ampicillin sodium

Fig -3: Thermal sample chromatogram



3.3. Identification and characterization of impurity by HRMS

The HRMS method, as described in materials and method was used to identify the unknown impurity (Fig 4,5,6). The m/z observed for impurity peak is 1066.3456 [M+H+]. To further investigate the chemical structure of the impurity, the sample was subjected to LCMS/ ESI QTOF. The high resolution mass analysis using Mass Lynx fragmentation tool proposed the following probable elemental compositions / molecular formulae with a mass error of 1.6 ppm [C48H60N9O13S3] (M+H+). Based on the high resolution mass fragmentation study, the chemical structure has been assigned to the impurity of m/z 1065.3378 eluting RRT 2.88. The structure of impurity was proposed as 3-[[(2R,3S)-[3-methoxy-3-N-[2-(thiophen-2-yl) acetamido]]-4-oxoazetidin-2-ylthio]-2-(carbam oyloxy)methyl]]-acrylic acids.(Trimer of ampicilloic acid)

Fig -4: Mass spectrum of Trimer Impurity

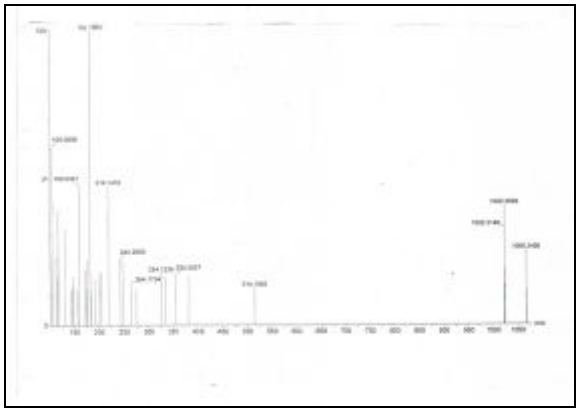
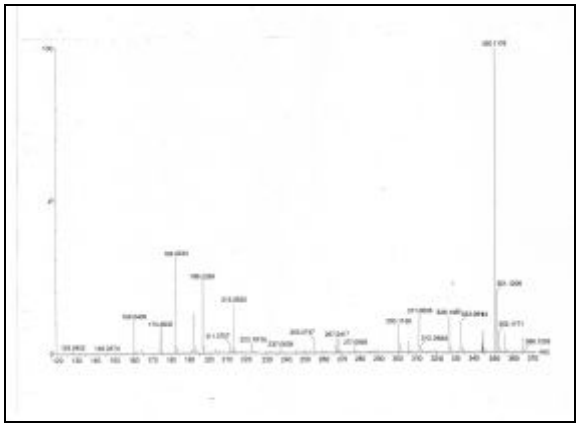


Fig -5: MS^2 spectrum of Trimer Impurity from



The MS2 spectra obtained for ampicillin showed prominent product ion peaks at m/z 514, m/z 381, m/z 324, m/z 191, m/z160 and m/z 106 (Fig.6). The fragment at m/z 1022can be attributed to the loss of CO2 (44 Da) as depicted in Fig 7. Fragmentation pattern of trimer of ampicilloic acids is matching with dimer of ampicilloic acid which is a well characterized impurity. Therefore the proposed structure of impurity is concluded as combination of oligomers of ampicilloic acids, as the entire major fragment ion peaks as observed in Dimer of ampicilloic acids, are observed in the impurity with m/z 1065.3378. The observed LCMS-QTOF major fragments of Trimer of Ampicilloic acids and of the impurity m/z 1065.3378 are shown in Table 1.

Table-1: Mass Fragments of Trimer of Ampilloic Acid

Name of the compound	LCMS-($M+H^+$)	Theoretical Mass ($M+H^{+}$)	Molecular Formula
Ampilloic Acid	1066.3456	1066.3473	$C_{48}H_{60}N_9O_{13}S_3$
Fragment 1	1022.3593	1022.3574	$C_{47}H_{60}N_9O_{13}S_3$
Fragment 2	514.1993	514.1947	$C_{25}H_{32}N_5O_3S_2$
Fragment 3	381.1538	381.1563	$C_{20}H_{21}N_4O_4$
Fragment 4	324.1239	324.1196	$C_{14}H_{18}N_3O_6$
Fragment 5	191.0845	191.0854	$C_7H_{15}N_2O_2S$
Fragment 6	160.0421	160.0432	$C_6H_{10}NO_2S$
Fragment 7	106.0659	106.0657	C_7H_8N

Fig -6: Mass spectrum Of Dimer impurity of Ampicillin

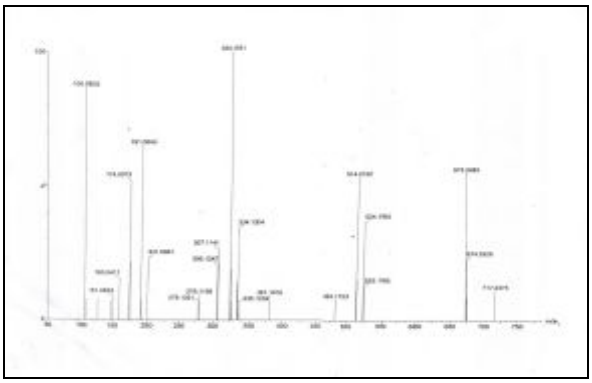
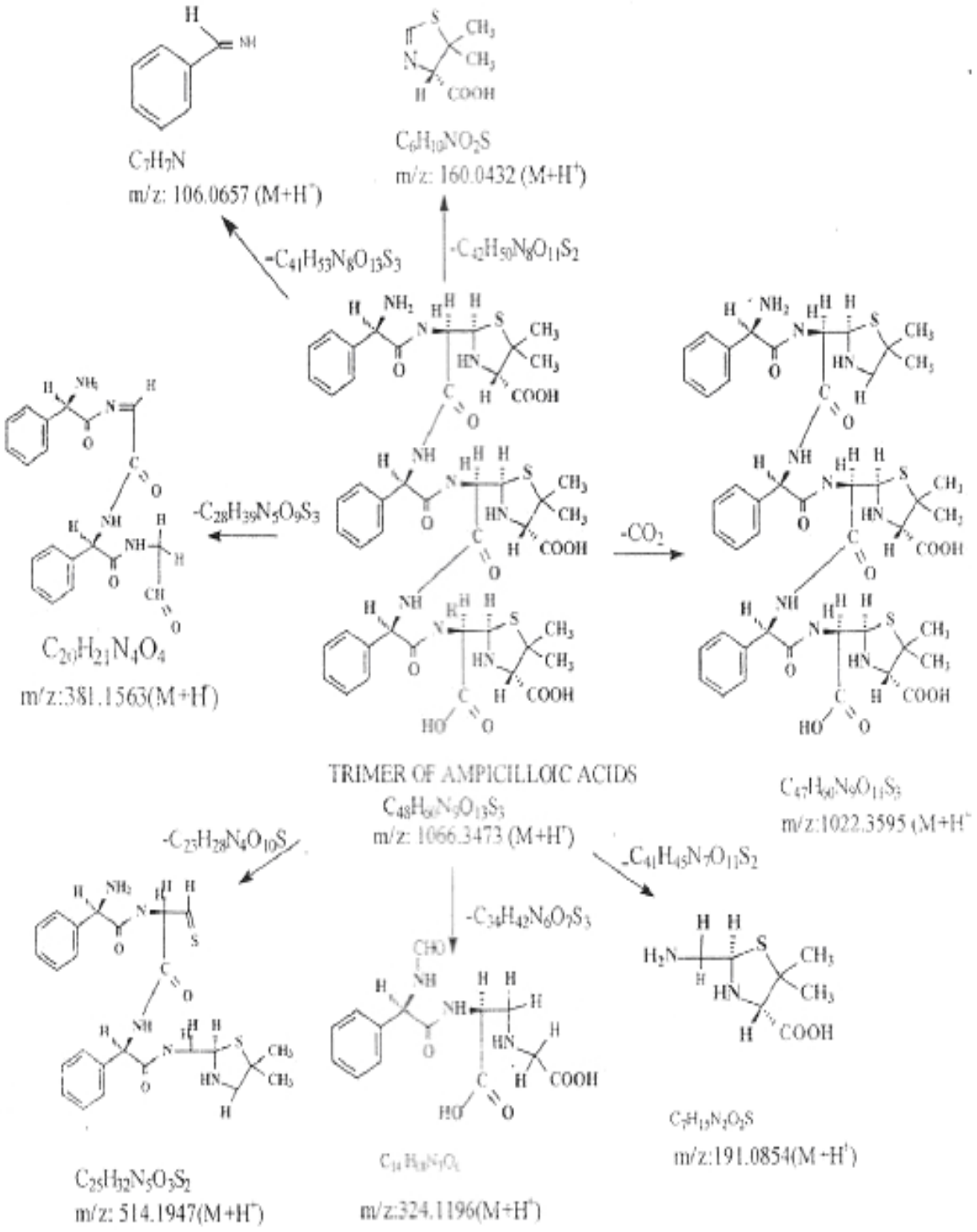


Fig -7: Mass Fragmentation Pattern of Trimer Impurity



3.4. Structural conformation by NMR

The NMR spectral data of Ampicillin sodium and impurity were compared and spectrum data has shown in (Table 2)

Table -2: Comparative NMR assignments for Ampicillin sodium and Trimer of Ampicilloic Acid

Spectral Data	Ampicillin Sodium	Trimer of Ampilloic Acid
^{1}H NMR	0.79, 1.0 (2s,6H, 19,20)	0.79, 1.0, 1.25 & 1.43 (2s,6H, 19,20)
	3.10(1s,1H, 5)	3.10 & 3.50 (2d,3H, 5, 27,49)
	4.42-5.48(m,3H,1,17,12)	4.42-.48(m,9H,1, 17, 12,23,9,34,45,61,56)
	7.34-7.41(m,5H, ArH)	7.34-7.41(m,15H, ArH)

Table -3: LOD and LOQ values of Trimer of Ampilloic Acid

Injection ID	Area of Trimer of Ampilloic Acid	Trimer of Ampilloic Acid
	LOD	LOQ
1	2916	6122
2	2625	6089
3	2154	6045
4	2725	6155
5	2254	6123
6	2916	6243
Mean	2535	6107
SD	322	42
%RSD	12.6	0.68
Conc (µg/mL)	0.163	0.326
Conc (%W/W)	0.013	0.025

3.5. LOD and LOQ determination of Trimer of Ampilloic Acid

The LOD and LOQ values of trimer of ampicilloic acids are determined based on the % RSD data, analyzing the diluted sample solutions and injected each solution six times into the HPLC. The concentration level that yielded RSD below 10.0% from the impurity peak area of six replicate injections is considered for LOQ and that below 33% as LOD.

4. CONCLUSION

In this study a potential degradant in Ampicillin sodium in Active pharmaceutical ingredients was identified. The impurity was isolated by semi-preparative liquid chromatography. The isolated impurity was characterized by using spectroscopic techniques. The above reported LOQ values for Trimer of ampicilloic acid was well below the specification level, indicating that the method is sufficiently precise for the quantification of the related substances also in ampicillin sodium drug substances.

5. REFERENCES

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