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Identification, isolation and structural characterization of unknown impurities in Cefdinir drug substance

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ABSTRACT

Two unknown impurities in Cefdinir drug substances at levels ranging from 0.1 to 0.35% were detected by High Performance Liquid Chromatography (HPLC). These impurities were isolated from Cefdinir drug substance using Preparative HPLC. Based on the spectral data's (UV, IR, NMR, MS) the structure of these impurities were characterized as (6R,7R)-7[(E)-2- (2-aminothiazol-4-yl)- 2-hydroxy iminoacetylamino]-8-oxo-3-vinyl-5-thia-1 -azabicyclo [4.2.0] oct-2- ene- 2carboxylic acid and (2E)-2-(2-amino-1, 3-thiazol-4-yl)-2-(hydroxyimino)-N-{[(2R)-5-methyl-7-oxo-1, 2, 5, 7-tetrahydro-4H-furo [3, 4-d] [1, 3] thiazin-2-yl] methyl}acetamide. By comparing the above structures defined for the unknown impurities with the structure of Cefdinir it was found that the basic nucleus of the impurities and the parent drug were similar, which concludes that the unknown impurities were the related substances of the Cefdinir drug substance. Monitoring of these impurities are useful for cefdinir drug development and quality control of cefdinir drug substance and its formulation. The impurity profiling of this compound has also been discussed.

Keywords: Antibacterial Drug, Cefdinir, Impurities, Synthesis, Identification, Isolation, Preparative HPLC, UV, IR, NMR and MS.

1. INTRODUCTION

Cefdinir is used to treat certain infections caused by bacteria such as bronchitis (infection of the airway tubes leading to the lungs) pneumonia and infections of the skin, ears, sinuses, throat, and tonsils. Cefdinir is in a class of medications called cephalosporin antibiotics. It works by killing bacteria. Antibiotics such as cefdinir will not work for colds, flu, or other viral infections. Using antibiotics when they are not needed increases your risk of getting an infection later that resists antibiotic treatment.The control of pharmaceutical impurities is currently a critical issue to the pharmaceutical industry. The pharmaceutical industry is required by the Food, Drug and Cosmetic Act to establish the identity and purity of all marketed drug products.

The United States Food and Drug Administration (FDA) and other regulatory bodies around the world require that impurities in drug substance and drug product when present at threshold levels recommended by the International Conference on Harmonization (ICH) be isolated and characterized.

Impurities in pharmaceuticals are the unwanted chemicals that remain with the active pharmaceutical ingredients (APIs), or develop during formulation, or upon aging of both API and formulated APIs to medicines. Therefore, identification, quantification, and control of impurities in the drug substance and drug product, are an important part of drug development and regulatory assessment.[1]

The Need to Isolate and Characterize Impurities

Any extraneous material present in the drug substance or active ingredient must be considered an impurity even if it is totally inert or has superior pharmacological properties, so that an appropriate evaluation of its content in the product can be made. According to ICH, the maximum daily dose qualification threshold is considered as follows: ≤ 2g/day 0.1 % or 1 mg per day intake (whichever is lower) ≥ 2g/day 0.05%.Therefore, the control of low level impurities is of great importance when a drug is taken in large quantities; for e.g, the use of

methotrexate (10-20g) to treat neoplasia or the use of vitamins as a fad, notably vitamin C.

Sources of Impurities

- Drug substance
- Inert ingredients used for Formulation
- Contact with packaging
- Solvents (eg. Water, Isopropyl alcohol, etc.,)
- Preparation of New chemical entity from synthetic raw materials
- Process parameters also produce impurities (eg. Heat used for drying)
- Originate during storage (shelf life) or shipment of drug products.

Separation Methods

- Chromatography
- Capillary electrochromatography
- Capillary electrophoresis
- Gas chromatography
- High-pressure liquid chromatography (HPLC)
- Supercritical fluid chromatography (SFC)
- TLC
- HPTLC

Isolation Methods

Isolation methods include both chromatographic and nonchromatographic methods. For isolation of a given compound from a complex mixture, the chromatographic methods utilized for separation of impurities in analytical determinations are the methods of first choice that are suitably modified for the purpose of isolation of impurities where an appropriate fraction is collected.

Characterization Methods

When an impurity has been detected, it becomes necessary to estimate its content. Adequate detectability frequently means that a given component provides a signal at least twice that of background noise or baseline noise. At times, the multiple is set higher for greater assurance of detectability. Initial estimations are generally done against the parent compound because in most cases the authentic sample of impurity is not available. It is important that the authentic sample be used for estimation when it's available. If the estimations indicate that a given impurity content is greater than 0.1%, it must be characterized according to FDA requirements.

The next step is to determine at what level the unknown impurities are present. Identification of impurities below the 0.1% level is generally not necessary unless the potential impurities are expected to be unusually potent or toxic. Therefore, it is imperative to determine the level of unknown impurity and/or degradation product early in the process. If the unknown is at or above the 0.1% limit, effort should be made to identity it. However, if the unknown is below the 0.1% threshold, a discussion should be conducted whether it is necessary to isolate and identify.

After a decision has been made to identify an unknown, the next logical step is to review all known process-related impurities, precursors, intermediates, and degradation products. A critical analysis of all available data can save considerable time and energy. By reviewing the HPLC retention data of all known process-related impurities, precursors and intermediates (if available), it can quickly be determined whether the impurity of interest is truly unknown. If the retention time of the unknown impurity matches that of a standard, the unknown can be identified by using HPLC-DAD-MS. The identity can be confirmed by correlating the retention time, UV spectra, and Mass spectra of the unknown impurity with that of the reference standard. Identifying an unknown by using a reference standard as in the preceding description is a quick and easy process. However, other steps must be taken when the retention time of an unknown does not match that of a standard.

The next step generally is to obtain molecular mass and fragmentation data via HPLC-MS. It is essential to determine the molecular mass of the unknown. Not only does the molecular mass help in the identification of the unknown, it also allows tracking the correct peak by HPLC when isolation becomes necessary. If such a method is not available, it has to be developed. The decisions regarding the best approach to characterizing impurities and degradation products should be based on sound reasoning in order to minimize the time and cost of new drug development.[2]

2. MATERIALS AND METHODS

Ammonium dihydrogen ortho phosphate (AR Grade), Acetonitrile (HPLC Grade), Liquor Ammonia (AR Grade, 25%), Acetic acid (AR Grade), Methanol (HPLC Grade), Milli Q water used for the research work.

Experimental Work

The following analytical HPLC conditions are developed In-House for the identification of cefdinir impurities.

a. Preparation of mobile phase

Mobile phase A

Accurately weigh 2.30g of ammonium dihydrogen ortho phosphate and dissolve in 1000 mL water and sonicate for 5 to 10 minutes to dissolve. Filter the solution through a membrane filter of 0.45 µ or finer porosity and degas.

Mobile phase B

Filtered and degassed Acetonitrile.

b. Preparation of diluent

Accurately weigh about 5.75g of ammonium dihydrogen ortho phosphate and dissolve in 1000mL water and sonicate for 5 to 10 minutes to dissolve. Adjust the pH to 7.1 ± 0.05 with 25% Liquor ammonia. . Filter the solution through a membrane filter of 0.45 µ or finer porosity and degas.

c. Chromatographic parameters

Flow Rate : 1.0mL/min

Column : Inertsil ODS-2 (250 X 4.6) mm, 5µ

Column Oven Temperature : 30ºC

Detection : UV at 247 nm

Injection Volume : 20µL

Run time : 70 min

Injection Delay : 10 min

Flow Rate : 1.0 mL/min

d. Elution program

Table No -1: Elution Program

Time (Minutes)	Mobile-A (% V/V)	Mobile-B (% V/V)
0.00	97.0	3.0
5.00	93.0	7.0
15.00	93.0	7.0
25.00	70.0	30.0
60.00	15.0	85.0
65.00	15.0	85.0
70.00	97.0	3.0

e. Preparation of Sample solution (Sample Concentration-1000ppm)

Accurately weigh and transfer about 50mg of cefdinir drug substance to a 50mL volumetric flask. Add 20mL of diluent and sonicate for 5 to 10 minutes to dissolve and makeup the volume with diluent. Filter the solution through a membrane filter of 0.45 µ or finer porosity and degas.

f. Procedure

Identification of Cefdinir Impurity-I And Impurity-II

1. Separately injected 20µL each of blank solution and standard solution (in duplicate)and sample solution into the analytical HPLC and record the Peak responses.
2. Examined the blank chromatogram for extraneous peak and disregard the corresponding peaks observed in the chromatogram of the sample solution.
3. Determined the peak responses of eluting peaks from the chromatographic report and Cefdinir related substances (unknown impurities) present in the sample was identified.

Forced Degradation Studies

Stress testing of the drug substance can helps to identify the likely degradation products, which can in turn help to establish the degradation pathways and the intrinsic stability-indicating power of the analytical procedures used. The nature of the stress testing depends upon the individual drug substance and the type of drug products involved.

As per the ICH Guidelines on impurities in New Drug Substances, the impurities above 0.1% should be isolated and characterized. Since the two impurities are in the range of 0.1-0.35%, it's very difficult to purify. So it was important to go on for forced degradation studies on cefdinir drug substance using acid, base, thermal, humidity, UV and H_2O_2 in order to monitor whether any possible enrichment of the required impurities in the cefdinir drug substance.

a. Acid degradation

100 milligrams of cefdinir was dissolved in 25 ml of diluent, 1ml of 0.1N HCl solution was added and kept for 3 days at room temperature.

b. Base degradation

100 milligrams of cefdinir was taken; add 5mL of water and the pH of the solution was adjusted to 12.7 by 2M NaOH. The above solution was kept at 50ºC for 30 minutes, cool and the pH was adjusted to 1.0 by 2M HCl. Then the solution was kept at room temperature for 3 days.

c. Peroxide degradation

100 milligrams of cefdinir was dissolved in 25 ml of diluent, 1ml of 3% hydrogenperoxide solution was added and kept for 3 days at room temperature.

d. Thermal degradation

100 milligrams of cefdinir drug substance was subjected to thermal degradation at 105°C for about 3days.

e. Humidity degradation

100 milligrams of cefdinir drug substance was subjected to humidity degradation for about 3days in the desiccator.

f. UV degradation

100 milligrams of cefdinir drug substance was subjected to UV degradation for about 3days kept in UV chamber.

Isolation of Cefdinir Impurity-I and Impurity-II

Development of Preparative Method[2]

Separation of the desired material from interfering bands in the sample is the primary objective when developing a preparative chromatographic method. The main objective of almost every chromatographic separation is to optimize system resolution, speed and capacity, otherwise known as sample load. Focusing on any one of these three parameters will require certain concessions from the other two. Sample load is usually the parameter compromised when developing an analytical separation, because peak recovery is not the primary goal of the separation. On the other hand, when component isolation is the main objective, emphasis should be on maximizing sample load, while speed and resolution will be compromised. It is advisable to invest more time exploring how selectivity varies with experimental conditions, because preparative separations can require large periods per run. Every effort should be made to perform loading studies with the same particle size and column length available at the preparative scale, as well as the same lot of feed material as the preparative separation.

Analytical HPLC separations performed with sample loads of <1mg/g of column packing material usually do not alter column effectiveness. Preparative separations involve sample loads >1mg/g of stationary phase, and in many cases, the higher loading results in reduced selectivity is of more importance in preparative chromatography than in analytical separations. Resolution (R_S) is affected by several chromatographic variables demonstrated in the following equation:

$R_S = ¼ (\alpha-1) \sqrt{N} (k'/ (1+k))$

where $k' = (t-t_o)/ t_o$; to= time for a peak eluting in the void fraction of the column; t = time for component of interest to elute; α = k' of desired compound/k' of nearest neighbor; N = number of plates.

Selectivity and retention are, for all intents and purposes, free of the effect of flow rate as long as mass-transfer rates are not limiting. Resolution is relatively predictable for columns operated at loads of <1mg/g of packing. In overloaded columns the large sample size significantly affects column equilibrium, and parameters such as plate count (N), retention (k'), and selectivity (α) change as overload varies. Deliberately overloading a column while maintaining an adequate separation decreases both selectivity and retention while increasing throughput.

To begin an overload study, the feed solubility will need to be determined. The feed material should be dissolved in mobile phase at increasing concentration until it begins to fall out of solution. A concentration that is 80% of the maximum solubility at room temperature is typically used to load the column. If the solubility in the mobile phase is relatively poor (<5-10 mg/mL), then alternate feed solution should be tried (i.e., dissolving in the stronger eluent, use of another solvent). However the effect of the change in solvent will have to be determined as the peak shapes often can be significantly affected, and in extreme cases, the feed solution can precipitate on the column frits leading to high back pressure.

Once a feed solution has been determined, the analytical-scale preparative system can now be run under increasing loading. The injection volume should be increased in a stepwise manner, and the resolution of the desired component from the nearest eluting peak(s) must be monitored. Often the UV absorption is over the reporting time at higher loadings, and a wavelength off from the maximum absorption should be used to monitor loading studies. It is common to perform loading studies with several stationary-phase/mobile-phase combinations to determine the most effective system. Systems can be compared by their throughput. Throughput is the maximum system loading in mass per column volume, divided by the run time for that system. The peak of interest from each system should also be collected during these loading studies, and analyzed to ensure that it is indeed resolved to the desired purity.

To isolate relatively small amounts of the desired impurity at high relative purity, the sample load should be increased until the base of the peak of interest begins to overlap with an adjacent peak(s). This methodology will give the highest purity with highest recovery of the peak of interest without having to worry about fractionating the product peak and recycling partially pure fractions. To isolate more materials

than can be done with an analytical column, simply scale up the separation based on the preparative column diameter to allow for larger sample mass introduction. In the separation just described, the capacity of the system is limited by the resolution required.

If larger amounts of purified materials are needed than can be separated using the technique above, the column loading will have to be increased beyond the toughing bands. The degree of peak overlap will increase with increasing sample load. By collecting eluant fractions through the region of the peak of interest, one can collect a greater amount of material in a shorter period. However, collecting material in this "heart-cut" portion of the chromatography will provide the desired component at lesser purity than that of the same component collected at the loading limit mentioned earlier and at a lower yield. Collecting subfractions through the heart-cut range can provide solutions that contain the component of interest at rather high purity. Subfractions that do not meet the purity requirement can either be discarded or repurified, depending on the scarcity of the feed material.

Employing heart-cut methodology allows for added flexibility, especially when the component of interest is hidden within a matrix of several peaks. For exceptionally difficult separations, it may be necessary to isolate the desired impurity as a mixture from the first column. It may be possible to repurify the mixture on the same column with a second pass, or alternatively, it may be necessary to screen this mixture for alternate columns that can provide resolution between the remaining components. Having to deal with only one or two interfering peaks will greatly increase the chance of success for isolation of the desired band.

After the desired bands are collected, it is likely that undesired components remain on the column. Obviously, if these materials elute rather quickly from the point at which the desired bands were collected, it is best to continue elution without increasing the strength of the solvent. This will avoid the need for equilibration before the next sample injection. In many cases, significant undesired components from the feed matrix elute at extreme concentration and/or retention. Increasing the flow rate can sometimes resolve this situation; again, undesirably high back pressure or an unnecessary increase in solvent usage can limit this. A better method to clear the column of undesired material is to sharply increase the solvent strength and then follow with a period of equilibration with the desired mobile phase. The column is then ready for another injection of the feed matrix or a second pass of the pooled material collected from the heart-cut fractions.

Scaleup of Preparative Method[2]

A. Column Sizing

Once loading studies have been performed, scale-up can take place. First, the amount of feed material needed to yield the desired quantity of impurity must be determined. An HPLC assay or other chemical assay can be used to determine the potency of the feed with respect to the desired impurity. If an assay is not available, the impurity potency can be approximated from analytical peak collection. Additionally, an estimation of the recovery yield can be obtained from a mass balance on the high-loading peak collection experiments. Off-cuts, fractions collected from the column that contain the impurity but do not meet purity, can often be recycled into the feed for the next run, increasing the recovery yield. The required preparative column volume can then be calculated as follows:

Where V = prep- scale column volume (L); M = mass of impurity needed (g); P = potency of column feed (%); Y = recovery yield (%) as determined in loading studies; L = maximum loading as determined in loading studies (g feed/ L column); and C = number of cycle to be run.

In order to maintain the same level of resolution and run time seen at the analytical scale, the column length should be held constant if possible. The column diameter can than the scaled to obtain the desired column volume.

Where D_P = prep - scale column diameter (cm); V = prep - scale column volume (mL); L = column length. (cm)

B. Flow Rate

To achieve the same level of separation at the large scale at the analytical scale, the linear velocity through the column should be maintained and the flow rate adjusted. This can be done by the following calculations

Where v_a= velocity at analytical scale (cm/min); Q_a = flow rate analytical scale (mL/ min); D_a = diameter of analytical column (cm)

The prep- scale flow rate is then determined

$$\mathbf{Q_P = V_a \times \pi \times (D_P/2)^2}$$

QP = flow rate at prep - scale (mL/min); D_P = diameter of preparative column

C. Particle Size

The same particle-size resin as used for the loading studies should be used for scaleup.

The choice of particle size is a trade- off between resolution, which decreases with an increase in particle size, and pressure drop, which also decreases with an increase in particle size. Often, bulk resins are not offered in the full range of particle sizes that analytical columns are. Additionally, a size should be selected so that the column pressure drop does not exceed the resin limitations. A summary of the effect of changing the operating conditions is shown in table.no 2.

PREPARATIVE HPLC WORK

Isolation of Cefdinir Impurity- I

On several trails the following conditions were optimized for the isolation of Impurity-I of maximum purity.

PREPARATIVE HPLC CONDITIONS

a. Preparation of mobile phase

Mobile phase A

0.1% Acetic acid (1mL of Acetic acid in 1000mL of water)

Mobile phase B

Filtered and degassed Methanol

b. Chromatographic parameters

Column : YMC-PACK ODS-AQ (500 x30) mm, 10µ

Flow rate : 40.0 mL /min

Detection : UV at 247 nm

Injection volume : 5mL

Run time : 90 min

c. Elution Programme

d. Preparation of Sample Solution

250mg of Cefdinir5Hrs Thermal Degraded Sample was taken in a 10mL vial and dissolved in 5 mL of diluent sonicate for 5 to 10 minutes to dissolve. Filter the solution through 0.45 µ or finer porosity membrane filter.

e. Procedure

The prepared sample solution was injected in Preparative HPLC and all the eluted peaks were collected by separate conical flask. The collected fractions were analyzed by analytical HPLC in In-house analytical conditions. The fraction which was >90% of Chromatographic purity and matches with the RT of Impurity I was taken and further confirmedby its mass. The required fraction was distilled to remove organic phase and lyophilized to obtain the solid. The purity of the solid was checked to identify whether any degradation arises due to distillation or lyophilization.

Table.No.2- Effects of changes in column conditions

Column conditions	Effects of changes			
	Change	Resolution	Run time	Pressure drop
Column length	↑	↑	↑	↑
Column diameter	↑	↔	↔	↔
Particle size	↑	↓	↓	↓
Linear velocity	↑	↓ (case dependent)	↓	↑
Column velocity	↑	↓	↔	↔

Table – 3: Elution Programme

	Time (Minutes)	Flow (mL)	Mobile-A (% V/V)	Mobile-B (% V/V)
1	0.00	40.00	100.0	0.0
2	20.00	40.00	95.0	5.0
3	50.00	40.00	90.0	10.0
4	75.00	40.00	85.0	15.0
5	90.00	40.00	85.0	15.0

Isolation of Cefdinir Impurity- II

On several trails the following conditions were optimized for the isolation of Impurity-II of maximum purity.

PREPARATIVE HPLC CONDITIONS

a. Preparation of mobile phase

Mobile phase A

Water

Mobile phase B

Filtered and degassed Acetonitrile

b. Chromatographic parameters

Column : YMC-PACK ODS-AQ (500 x30) mm, 10µ

Flow rate : 50.0 mL /min

Detection : UV at 247 nm

Injection volume : 5mL

Run time : 70 min

c. Elution Programme

Table - 4: Elution Programme

Time (Minutes)	Flow (mL)	Mobile-A (% V/V)	Mobile-B (% V/V)
0.00	50.00	100.0	0.0
15.00	50.00	95.0	5.0
30.00	50.00	90.0	10.0
50.00	50.00	85.0	15.0
70.00	50.00	80.0	20.0

d. Preparation Sample Solution

250mg of CefdinirBase Degraded Sample was taken in a 10mL vial and dissolved in 5 mL of diluent sonicate for 5 to 10 minutes to dissolve. Filter the solution through 0.45 µ or finer porosity membrane filter.

e. Procedure

The prepared sample solution was injected in Preparative HPLC and all the eluted peaks were collected by separate conical flask. The collected fractions were analyzed by analytical HPLC in In-house analytical conditions. The fraction which was >95% of Chromatographic purity and matches with the RT of Impurity II was taken and further confirmedby its mass. The isolated fraction of Impurity-II was distilled to remove organic phase and lyophilized to obtain the solid. The purity of the solid was checked to identify whether any degradation arises due to distillation or lyophilization.

Structural Characterization of Cefdinir Impurity-I and Impurity - II

Characterization of cefdinir impurities were performed and confirmed by its spectral data's obtained from NMR, MS, IR, and UV.

UV spectroscopy

UV spectrum was recorded in Shimadzu UV-2550 Model using water as medium which operates in UV probe software.

IR Spectroscopy

The IR spectra for Cefdinir, impurity-I, II were recorded as KBr pellet using Perkin-Elmer spectrum one FT-IR spectrophotometer.

NMR spectroscopy

The 1H NMR, 13C NMR (proton decoupled) and DEPT spectra were recorded on Bruker Avance 400 MHz Spectrometer at 25^{o}C using DMSO-d_6 as solvent and Tetramethylsilane (TMS) as internal standard.

Mass spectroscopy

The mass spectrum of the isolated degradation product was acquired on PE Sciex API 3000 mass spectrometer using Analyst software for data acquisition and data processing. The analysis was performed in positive ionization mode with Turbo Ion Spray interface at 450^{o}C with the following conditions such as Ion source voltage 5200V, declustering potential 10V and entrance potential 10V with the nebuliser gas as nitrogen at 15 psi. The MS–MS data was generated with the collision energy ramping from 30 to 60V and the Mass range was scanned between 100 and 1100 amu.

3. Results and Discussion

RESULTS AND DISCUSSION

Identification of Cefdinir Impurities

The analytical chromatogram of blank was recorded for extraneous peaks and disregard the corresponding peaks in all the chromatograms. From the analytical chromatograph of cefdinir, five different peaks arises at different RT have been recorded. The peak at RT 17.513 is the cefdinir peak and the other four peaks at levels ranging from 0.1 to 0.35 % at RT 8.644, 14.702, 24.114, 28.512 are the unknown impurities. In this project the unknown impurities at RT 24.114 corresponds to 1.37 RRT as Impurity-I and RT 28.512 corresponds to 1.62 RRT as Impurity-II have been taken for its isolation and structural characterization.

Forced Degradation Studies

Base degradation study shows that Impurity-II enriches from 0.1% to 6.5 % and the thermal degradation study shows that the Impurity-I enriches from 0.3% to 2%. Base degraded sample enriched to 6.5% was chosen for the isolation of Impurity-II and further studies were performed in thermal degradation to obtain further enrichment of Impurity-I, since the level of Impurity-I was only 2%.

The spiking of cefdinir sample with the base degraded sample and the thermal degraded sample respectively, which confirms that the enriched impurities at RT 28.51 and RT 24.08 as

the required impurities to be isolated and found matching with the required RRT at 1.61 and 1.34.

The thermal degradation studies were conducted in other possible environments and conditions to obtain further enrichment of Impurity-I. A study on Cefdinir sample dissolved in diluent and kept in oven at 90°c shows an increase in the level of Impurity-I gradually with increase in time. The thermal degradation of the cefdinir drug substance dissolved in diluent and kept in oven at 90°c for 1Hr, 3Hrs and 5Hrs.

The thermal degradation for 1Hr, 3Hrs and 5Hrs shows the level of Impurity-I gradually increases to 4%, 13% and 19% respectively with the increase in the level of other peaks which makes difficult to isolate and therefore, further degradation of the sample was stopped and the thermal degraded sample for 5Hrs at 90°c kept in oven increased to 19% was chosen for Isolation of Impurity-I. The enriched impurity was confirmed by spiking it along with the sample and found matching with the required RRT 1.39.

Table – 5: Results of Forced Degradation Studies Observed from the Chromatogram

Degradation methods	Impurity-i	Impurity-ii
Acid degradation	-	-
Base degradation	-	**6.5%**
H_2O_2 degradation	-	-
Thermal degradation	**2%**	-
Humidity degradation	-	-
UV degradation	-	-

Table - 6: Thermal Degradation of Cefdinir Dissolved in Diluent and kept in Oven at 90°c

TIME	% ENRICHMENT
1Hr	4% of Impurity-I
3Hrs	13% of Impurity-I
5Hrs	19% of Impurity-I

Isolation of Cefdinir Impurity-I and Impurity-II

Isolation of Impurity-I

The fraction collected at 73.8-82.3 minutes in the preparative was analyzed in the analytical HPLC condition and the analytical chromatogram shows the elution of peak at the required RT 24.07 and its purity was found to be 95%. It is confirmed as Impurity-I by spiking the preparative fraction with the cefdinir sample and found matching with the required RRT 1.38. It is further confirmed by its mass number that the mass of the collected fraction was 396 g/mol which is similar to Impurity-I. The purity of the isolated solid Impurity-I after distillation and lyophilization was analyzed and found to be 95%**.**

Isolation of Impurity-II

The fraction collected at 43.7-47.8 minutes in the preparative was analyzed in the analytical HPLC condition and the analytical chromatogram shows the elution of peak at the required RT 28.53 and its purity was found to be 96%. It is confirmed as Impurity-II by spiking the preparative fraction with the cefdinir sample and found matching with the required RRT 1.65. It is further confirmed by its mass number, that the mass of the collected fraction was 396 g/mol which is similar to Impurity-II are 369.The purity of the solid isolated Impurity-II after distillation and lyophilization was analyzed and found to be 96%**.**

Finally the isolated solid Impurity-I and Impurity-II was spiked with Cefdinir and its purity and RRT was confirmed.

Structural Characterization of Cefdinir Impurity - I and Impurity - II

Structural Characterization of Impurity-I

UV spectral studies

UV spectrum was recorded in water as medium**.** The λmax at 287 nm indicates the presence of groups responsible for n →π* transition of carbonyl group in addition to π →π*.

IR spectral studies

IR spectrum was recorded using KBr pellet technique. A strong absorption in the region at around 3350 cm^{-1} showed the presence of OH group in the molecule. The absorption at 1763cm^{-1} indicates that the carbonyl stretching of β-lactum moiety in the molecule.

The IR stretching frequencies were assigned and are listed in table

Table – 7: IR Specral Assignment of Cefdinir Impurity –I

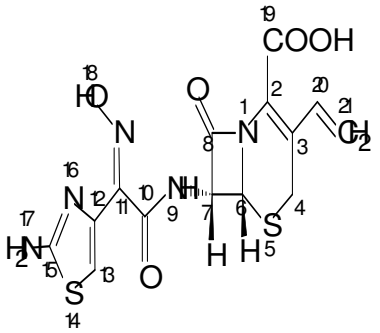
Wave numbers (cm^{-1})	Assignment
~3350	O-H stretching
3000-2800	C-H stretching
~1763	C=O stretching (β-lactum)
~1650-1510	Amide-I and Amide-II bands
1300-1100	C-O stretching
900-670	Out of plane amide N-H wagging

NMR spectral studies

^{1}H NMR studies

^{1}H NMR spectrum was recorded in DMSO-d_6 solvent**.** The two doublet at δ 3.48 ppm and 3.75 ppm corresponds to 4-CH_2 with geminal coupling at 17.6 H_Z. The doublet atδ 5.10 ppm corresponds to 6-CH with vicinal coupling constant 4.8 H_Z. The two doublets atδ 5.23 and 5.50 ppm correspond to 21-CH_2 with coupling constant of 17.5 H_Z and 11.4 H_Z. The doublet of doublets at δ 5.72 ppm correspond to7-CH with coupling constant of 8.3 H_Z and 4.8 H_Z. The singlet at 7.44 ppm corresponds to 13-CH.

All the ^{1}H signals were assigned on the basis of their chemical shift and coupling constant values and were listed in the table.



Chemical shift at δ 2.50 and 3.40 ppm were due to solvent DMSO-d_6 and solvent water respectively.

^{13}C NMR studies

^{13}C NMR spectrum was recorded in DMSO-**.** All the ^{13}C signals were assigned on the basis of their chemical shift values and were listed in the table.

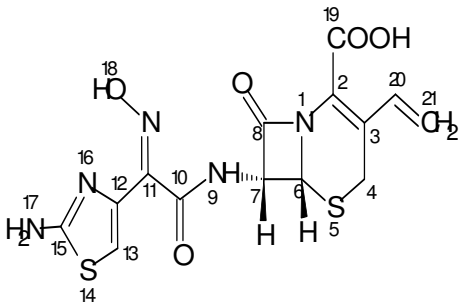


Table – 8: ^{1}H NMR CHARACTERISTICS OF CEFDINIR IMPURITY-I IN DMSO-d_6

Chemical shift δ (ppm)	Multiplicity	Number of Hydrogen(s)	Coupling constant $^{2}J_{HH}$, $^{3}J_{HH}$&$^{4}J_{HH}$ (H_Z)	Assignment
3.48 & 3.75	Two doublets	2	17.6	4-CH_2
5.10	Doublet	1	4.8	6-CH
5.23 & 5.50	Two Doublets	2	17.5 & 11.4	21-CH_2
5.72	Doublet of doublets	1	8.3 & 4.8	7-CH
6.85	Doublet of doublets	1	17.5 & 11.4	20-CH
7.13	Broad signal	2	-	17-NH_2
7.44	Singlet	1	-	13-CH
9.33	Doublet	1	8.3	9-NH
12.52	Broad signal	1	-	18-OH

Table – 9: ^{13}C NMR Characteristics of Cefdinir Impurity-I in DMSO-d_6

Group/Carbon	Chemical shift in ppm (δ)
C-4	24.0
C-6	58.5
C-7	59.9
C-13	115.1
C-21	118.0
C-3	125.2
C-2	126.6
C-20	132.9
C-12	139.6
C-15	146.2
8-C=O	164.2
10-C=O	164.9
11-C=N	165.4
19-C=O	167.5

Chemical shift around δ 39.5 ppm was due to solvent DMSO-d_6.

DEPT 135 NMR studies

In ^{13}C NMR, methyl, methylene and methine signals were identified by using DEPT-135° spectrum.

The two negative signals in DEPT-135° experiment showed the presence of two methylene carbons. The four positive signals correspond to four methine carbons. The eight extra signals in ^{13}C NMR compared with DEPT-135° experiment correspond to eight quaternary carbons.The quaternary carbons were assigned on the basis of ^{13}C chemical shift additivity parameters.

Mass Spectral studies

MS data was acquired in positive ionization in FIA mode.

The molecular mass of the compound was identified as 395 from the mass spectrum. The mass number showed the presence of odd number of nitrogen atoms in the molecule. Additional evidence for the molecular mass was obtained from the m/z peak at 418 $[M+Na]^+$.

Mass spectral assignments were listed in the table.

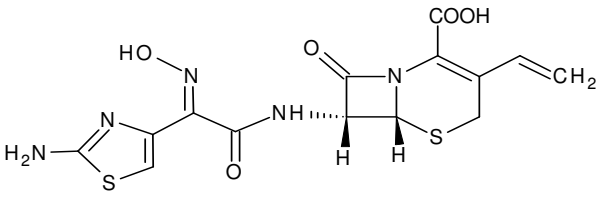


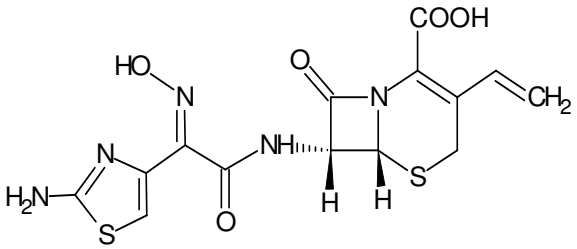
Table – 10: Mass Spectral Assignment of Cefdinir Impurity-I

m/z	Assignment
418	$[M+Na]^+$
398	Isotope peak of 396
396	$[M+H]^+$

Confirmed Structure

On the basis of spectral studies discussed above the structure of the molecule was confirmed and shown below:

1. Name of Impurity : Cefdinir Impurity-I
2. Description : A yellow Powder
3. RRT : 1.37
4. Molecular formula : $C_{14}H_{13}N_5O_5S_2$
5. Molecular weight : 395.41 g/mol
6. Structure :



(6R, 7R)-7[(E)-2-(2-Aminothiazol-4-yl)-2-hydroxyiminoacetylamino]-8-oxo-3-vinyl-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid **(*E-Isomer*)**

Structural Characterization of Impurity-II

UV spectral studies

UV spectrum was recorded in water as medium. The λmax at 225nm indicates the presence of groups responsible for π →π* transitions in the molecule.

IR spectral studies

IR spectrum was recorded using KBr pellet technique. A strong absorption in the region at around 3414 cm^{-1} showed the presence of OH & NH stretching in the molecule. The absorption at 1744cm^{-1} showed the carbonyl stretching of lactone moiety in the molecule. The IR stretching frequencies were assigned and were listed in the table.

Table - 11: IR SPECRAL ASSIGNMENT OF CEFDINIR IMPURITY -II

Wave numbers (cm^{-1})	Assignment
~3414	O-H & N-H stretching
~1744	C=O stretching (Lactone)
~1654	-C=O stretching (Amide-I band)
~1532	-N-H bending(Amide-II band)
~1300-1100	C-O stretching

NMR spectral studies

^{1}H NMR studies

^{1}H NMR spectrum was recorded in DMSO-d_6. The doublet at δ 1.45 ppm corresponds to 22-CH_3 with vicinal coupling of 6.5H_Z and the splitting pattern and the number of protons showed the presence of –CH-CH3 group. The absence of vinyl proton showed there was a change in the molecular environment at this position when compared with cefdinir. The D_20 exchange study confirmed the presence of five exchangeable protons in the molecule and the corresponding ^{1}H NMR spectrum.

All the ^{1}H signals were assigned on the basis of their chemical shift and coupling constant values and were listed in the table.

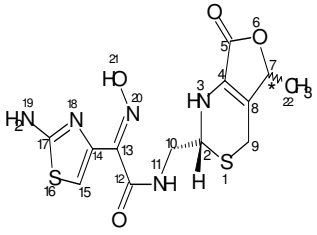


Table - 12: ^{1}H NMR CHARACTERISTICS OF CEFDINIR IMPURITY-II IN DMSO-d_6

Chemical shift δ (ppm)	Multiplicity	Number of Hydrogen(s)	Coupling constant $^2J_{HH}$ &$^3J_{HH}$ (H_Z)	Assignment
1.34	Doublet	3	6.5	22-CH_3
3.44	Multiplet	2	-	10-CH_2
3.53	Multiplet	2	-	9-CH_2
4.51	Multiplet	1	-	2-CH
5.06	Quartet	1	6.5	7-CH
6.10	Two Doublets	1	5.6	3-NH
7.28	Broad singlet	2	-	19-NH_2
7.47	Singlet	1	-	15-CH
8.56	Doublet	1	-	11-NH
13.01	Singlet	1	-	21-OH

Chemical shift at δ 2.50 and 3.39 ppm were due to solvent DMSO-d_6 and solvent water respectively.

^{13}C NMR studies

^{13}C NMR spectrum was recorded in DMSO-d_6. All the ^{13}C signals were assigned on the basis of their chemical shift values and were listed in table.

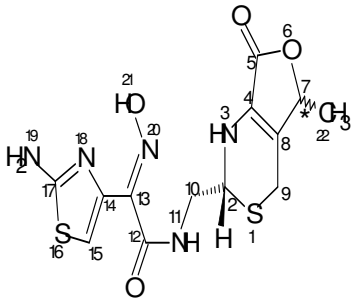


Table.No.13. ^{13}C NMR characteristics of cefdinir impurity-II IN DMSO-d_6

Group	Chemical shift in ppm (δ)
C-22	19.8 & 19.9
C-9	22.5 & 22.8
C-10	42.2 & 42.3
C-2	57.4 & 57.8
C-7	79.3
C-15	114.6
C-8	129.4 & 129.5
C-4	129.7 & 129.8
C-14	139.7
13-C=N	146.0
12-C=O	163.6
17-C=N	167.8
5-C=O	169.9

Chemical shift at δ 39.5 ppm was due to solvent DMSO-d_6.

DEPT 135 NMR studies

In ^{13}C NMR, methyl, methylene, methane, aryl and quaternary signals were identified by using DEPT-135° spectrum and was displayed in **figure-37**.

The two sets of negative signals in DEPT-135° experiment showed the presence of two sets of methylene carbons corresponding to two diastereoisomers. The four sets of positive signals corresponding to one set of methyl, two sets of methine carbons, and one set of aryl carbon corresponding to two diastereoisomers. The seven extra signals in ^{13}C NMR compared with DEPT-135° experiment correspond to seven quaternary carbons. The quaternary carbons carbons were assigned on the basis on the of ^{13}C chemical shift additivity parameters.

Mass Spectral studies

MS data acquired in positive ionization FIA mode and was displayed in **figure-38**.

The molecular mass of the compound was identified as 369 from the mass spectrum. The mass number showed the presence of odd number of nitrogen atoms in the molecule. Additional evidence for the molecular mass was obtained from the m/z peak at 392 $[M+Na]^+$.

Mass spectral assignments were listed in the table

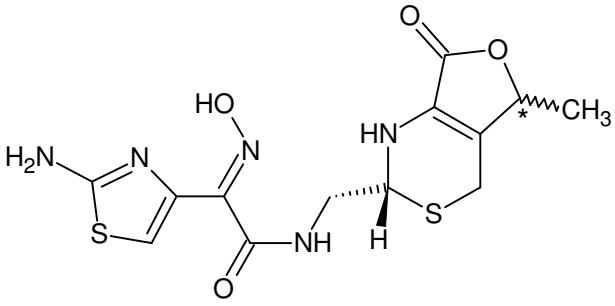
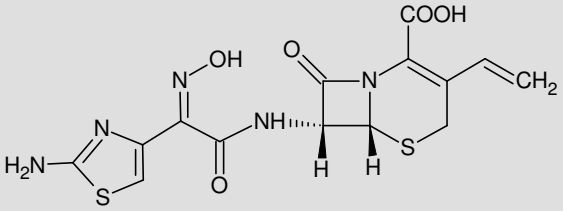
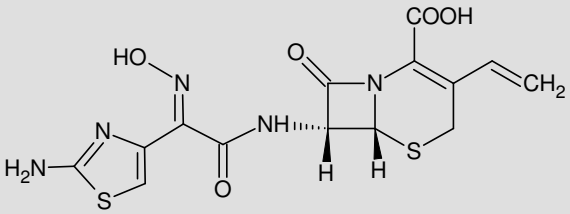
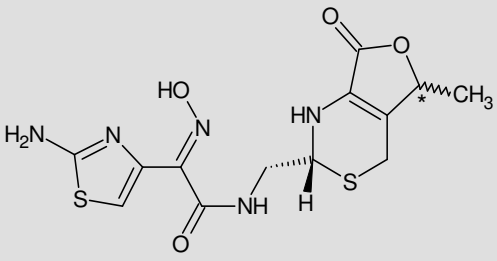


Table - 14: Mass spectral assignments of cefdinir impurity-II

m/z	Assignment
408	$[M+K]^+$
392	$[M+Na]^+$
370	$[M+H]^+$

Table – 14: Comparision of Unknown Impurities

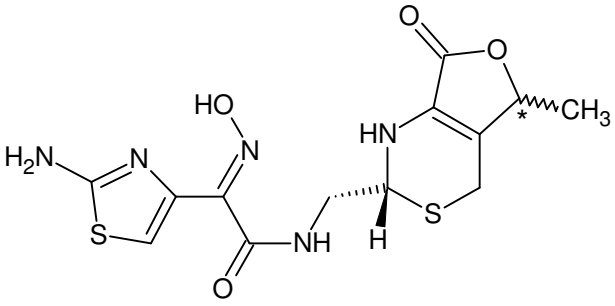
Name	Molecular weight (g / mol)	Relative Retention Time (RRT)	Structure
Cefdinir	396	-	
Impurity -I	396	1.37	
Impurity - II	369	1.62	

The basic nucleus of the unknown impurities matches with the Cefdinir drug substance.

Confirmed structure

On the basis of spectral studies discussed above the structure of the molecule was confirmed and was shown below:

1. Name of Impurity : Cefdinir Impurity-II
2. Description : yellow colour solid
3. RRT : 1.62
4. Molecular formula : $C_{13}H_{15}N_5O_4S_2$
5. Molecular weight : 369.42 g/mol
6. Structure :



(2E)-2-(2-amino-1,3-thiazol-4-yl)-2-(hydroxyimino)-N-{[(2R)-5-methyl-7-oxo-1,2,5,7-tetrahydro-4*H*-furo[3,4-d][1,3]thiazin-2-yl]methyl}acetamide ***(Diastereoisomers-I&II)***

Comparision of Unknown Impurities with Cefdinir Drug Substance

Based on the above studies on the isolation and characterization of the Cefdinir Impurity I and II - Mass number, Relative retention Time and structure of the Cefdinir impurities were defined as follows.

Conclusion

Two unknown impurities in Cefdinir drug substance at levels ranging from 0.1 to 0.35 % have been detected by High Performance Liquid Chromatography. These impurities were isolated from Cefdinir drug substance using Preparative HPLC.

Based on the spectral data's (UV, IR, NMR, MS) the structure of these impurities were characterized as (6R,7R)-7[(E)-2- (2-aminothiazol-4-yl)- 2-hydroxy iminoacetylamino]-8-oxo-3-vinyl-5-thia-1 -azabicyclo [4.2.0] oct-2- ene- 2carboxylic acid and (2E)-2-(2-amino-1, 3-thiazol-4-yl)-2-(hydroxyimino)-N-{[(2R)-5-methyl-7-oxo-1, 2, 5, 7-tetrahydro-4H-furo [3, 4-d] [1, 3] thiazin-2-yl] methyl}acetamide.

By comparing the above structures defined for the unknown impurities with the structure of Cefdinir it was found that the basic nucleus of the impurities and the parent drug were similar, which concludes that the unknown impurities were the related substances of the Cefdinir drug substance.

Monitoring of these impurities are useful for cefdinir drug development and quality control of cefdinir drug substance and its formulation.

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