4.1 METHODOLOGY

4.1.1 HPLC METHOD FOR ASSAY OF CEFTRIAXONE IN CEFTRIAXONE SODIUM FOR INJECTION

A) PREPARATION OF SOLUTIONS

Preparation of 1N sodium hydroxide

Dissolved about 4.1gm of sodium hydroxide in 100ml in purified water.

Preparation of 20% w/v ortho phosphoric Acid

Weighed 25.0gm of ortho phosphoric Acid (80% pure) and diluted to 100ml with purified water.

Preparation of 0.02M phosphate buffer

Weighed and dissolved 2.8gm of anhydrous disodium hydrogen ortho phosphate in 500ml purified grade water and diluted to 1000ml with purified water.

Preparation of 0.4% w/v tetraheptyl ammonium bromide

Weighed and dissolved 4.0gm of tetraheptyl ammonium bromide in 500ml of acetonitrile and diluted to 1000ml with acetonitrile.

Preparation of mobile phase

Measured 300mL of 0.02M phosphate buffer, 250ml of 0.4%w/v tetraheptyl ammonium bromide, 2.2ml solution of 20%w/v ortho phosphoric acid and mixed well. Adjusted pH between 6.6 and 6.8 using 20%w/v ortho phosphoric acid and 1N sodium hydroxide. Degassed and filtered through 0.45µ membrane filter.
**Preparation of resolution solution**

Accurately weighed and transferred about 15.8mg each of Ceftriaxone Sodium working standard and Ceftriaxone E-isomer in a 100mL volumetric flask, shaken well to dissolve and diluted to volume with mobile phase.

**Preparation of standard solution**

Accurately weighed and transferred about 25.2mg of Ceftriaxone Sodium working standard into a 100mL volumetric flask, shaken well to dissolve and diluted to volume with mobile phase.

**Preparation of sample solution**

Method validation sample solutions - Accurately weighed and transferred about 30.1mg of Ceftriaxone sodium for injection in to 100mL volumetric flask, shaken well to dissolve and diluted to volume with mobile phase.

Forced degradation sample solutions – Forced degradation samples are neutralized after treatment of the sample in acidic and basic conditions.

Stressed sample solutions are further diluted with mobile phase to get a final concentration of 0.2mg/ml.

Reconstitution samples solutions – Sample solutions reconstituted by using different diluents and diluted further to get final concentration 0.2 mg/ml.
B. CHROMATOGRAPHIC CONDITIONS

Column : Hypersil, BDS C18, 150 x 4.6mm, 5µ , Make: Thermo

Flow rate : 1.0ml /min.

Detector : UV-VIS

Wavelength : 254nm

Column temperature : 30°C

Injection Volumes : 20µl.

Run Time : 24min

Injections

Separately injected 20 µL of blank (mobile phase), resolution solution, standard solution (five replicates) and sample solutions into the Waters HPLC and analysed the peak areas of Ceftriaxone using the Empower software II.

System suitability

Resolution between Ceftriaxone and Ceftriaxone E-isomer from resolution solution was verified. Tailing factor and theoretical plates of Ceftriaxone peak was verified from standard solution. %Relative standard deviation (RSD) for five replicate injections of standard was verified from standard solution.
4.1.2 HPLC METHOD OF ANALYSIS FOR DETERMINATION OF RELATED
SUBSTANCES IN CEFTRIAXONE SODIUM FOR INJECTION

A. PREPARATION OF SOLUTIONS

Preparation of 1N sodium hydroxide
Dissolved about 4.3gm of sodium hydroxide in 100ml in purified water.

Preparation of 20% w/v ortho phosphoric Acid
Weighed 25.5gm of ortho phosphoric Acid (80% pure) and diluted to 100ml with purified
water.

Preparation of 0.02M phosphate buffer
Weighed and dissolved 2.9gm of anhydrous disodium hydrogen ortho phosphate in 500ml
purified grade water and diluted to 1000ml with purified water.

Preparation of 0.4% w/v tetraheptyl ammonium bromide
Weighed and dissolved 4.1gm of tetraheptyl ammonium bromide in 500ml of acetonitrile and
diluted to 1000ml with acetonitrile.

Preparation of mobile phase
Measured 300mL of 0.02M phosphate buffer, 250ml of 0.4%w/v tetraheptyl ammonium
bromide, 2.2ml solution of 20%w/v ortho phosphoric acid and mixed well. Adjusted pH
between 6.6 and 6.8 using 20%w/v ortho phosphoric acid and 1N sodium hydroxide.
Degassed and filtered through 0.45µ membrane filter.

Preparation of resolution solution
Accurately weighed and transferred about 15.8mg each of Ceftriaxone Sodium working
standard and Ceftriaxone E-isomer in a 100mL volumetric flask, shaken well to dissolve and
diluted to volume with mobile phase.
Preparation of standard solution

Accurately weighed and transferred about 25.2mg of Ceftriaxone Sodium working standard into a 100mL volumetric flask, shaken well to dissolve and diluted to volume with mobile phase.

Diluted 1 ml of this solution further to 100 ml with mobile phase.

Preparation of sample solution

Method validation sample solutions - Accurately weighed and transferred about 30.1mg of Ceftriaxone sodium for injection in to 100mL volumetric flask, shaken well to dissolve and diluted to volume with mobile phase.

Forced degradation sample solutions – Forced degradation samples are neutralized after treatment of the sample in acidic and basic conditions.

Stressed sample solutions are further diluted with mobile phase to get a final concentration of 0.2mg/ml.

Reconstitution samples solutions – Sample solutions reconstituted by using different diluents and diluted further to get final concentration 0.2 mg/ml.
B. CHROMATOGRAPHIC CONDITIONS

Column : Hypersil, BDS C18, 150 x 4.6mm, 5µ, Make: Thermo

Flow rate : 1.0ml/min.

Detector : UV-VIS

Wavelength : 254nm

Column temperature : 25°C

Injection Volumes : 20µl.

Run Time : 40min

Injections

Separately injected 20 µL of blank (mobile phase), resolution solution, standard solution and sample solutions into the Waters HPLC and analysed the peak areas of Ceftriaxone and its impurities using the Empower software II.

System suitability

Resolution between Ceftriaxone and Ceftriaxone E-isomer from resolution solution was verified.
4.1.3 HPLC METHOD FOR ASSAY OF CEFOTAXIME IN CEFOTAXIME SODIUM FOR INJECTION

A. PREPARATION OF SOLUTIONS

Preparation of buffer

Weighed 2.8 gm of disodium hydrogen orthophosphate anhydrous (Na₂HPO₄) and 1.4gm potassium dihydrogen orthophosphate anhydrous (KH₂PO₄) in 1000 ml of purified water, dissolved and mixed well.

Preparation of mobile phase

Mobile phase prepared by mixing the above buffer, methanol and acetonitrile in the ratio of 80:15:05. Adjusted the pH between 5.9 to 6.1 with diluted ortho phosphoric acid.

Degassed and filtered through 0.45µ membrane filter.

Preparation of resolution solution

Weighed and transferred accurately 1.2mg of each Cefotaxime sodium working standard and impurity-B into 10 ml of volumetric flask; dissolved the material in 1 ml of 1N hydrochloric acid (diluted 8.5 ml of concentrated hydrochloric acid to 100 ml with HPLC grade water) and 2 ml of acetonitrile. Diluted the solution to the volume with mobile phase and mixed well.

Preparation of standard

Weighed and transferred accurately 26.3mg of Cefotaxime sodium working standard into 100 ml volumetric flask; dissolved and diluted up to the volume with mobile phase and mixed well.

Preparation of sample solution

Method validation sample solutions - Accurately weighed and transferred about 24.9mg of Cefotaxime sodium for injection into 100mL volumetric flask, shaken well to dissolve and diluted to volume with mobile phase.
Forced degradation sample solutions – Forced degradation samples are neutralized after treatment of the sample in acidic and basic conditions.

Stressed sample solutions are further diluted with mobile phase to get a final concentration of 0.25mg/ml.

Reconstitution samples solutions – Sample solutions reconstituted by using different diluents and diluted further to get final concentration 0.25 mg/ml.

**B. CHROMATOGRAPHIC CONDITIONS**

- **Column**: Symmetry ODS, 150mm X 4.6mm, 5µ, Make: Waters
- **Column temperature**: 25°C
- **Flow rate**: 1.3 ml/min
- **Wavelength**: 254nm
- **Injection volume**: 20µl
- **Run time**: 10 minutes

**Injections**

Separately injected 20 µL of blank (mobile phase), resolution solution, standard solution (five replicates) and sample solutions into the Waters HPLC and analysed the peak areas of Cefotaxime using the Empower software II.

**System suitability**

Resolution between Cefotaxime and impurity-B from resolution solution was verified. Tailing factor and theoretical plates of Cefotaxime peak was verified from standard solution. %Relative standard deviation (RSD) for five replicate injections of standard was verified from standard solution.
4.1.4 HPLC METHOD FOR RELATED SUBSTANCES OF CEFOTAXIME IN CEFOTAXIME SODIUM FOR INJECTION

A. PREPARATION OF SOLUTIONS

Preparation of buffer solution

Weighed and dissolved 7.1 g of disodium hydrogen orthophosphate in 1000 ml of purified water and adjusted the pH in between 6.2 to 6.3.

Preparation of mobile phase A:

Mobile phase-A prepared by mixing the above buffer and methanol in the ratio of 88:12; degassed and filtered through 0.45µ membrane filter.

Preparation of mobile phase B:

Mobile phase-B prepared by mixing the above buffer and methanol in the ratio of 60:40; degassed and filtered through 0.45µ membrane filter.

Preparation of resolution solution

Weighed and transferred accurately 1.2mg of each Cefotaxime sodium working standard and impurity-B into 10 ml of volumetric flask; dissolved the material in 1 ml of 1N hydrochloric acid (diluted 8.5 ml of concentrated hydrochloric acid to 100 ml with HPLC grade water) and 2 ml of acetonitrile. Diluted the solution to the volume with mobile phase and mixed well.

Preparation of standard

Weighed and transferred accurately 42.1mg of Cefotaxime sodium working standard into 50 ml volumetric flask; dissolved and diluted up to the volume with mobile phase-A and mixed well.

Diluted 2 ml of this solution further to 100 ml with mobile phase-A and further 2.5ml of solution diluted to 25ml with mobile phase-A.
**Preparation of sample solution**

Method validation sample solutions - Accurately weighed and transferred about 41.3mg of Cefotaxime sodium for injection in to 50mL volumetric flask, shaken well to dissolve and diluted to volume with mobile phase.

Forced degradation sample solutions – Forced degradation samples are neutralized after treatment of the sample in acidic and basic conditions.

Stressed sample solutions are further diluted with mobile phase to get a final concentration of 0.8mg/ml.

Reconstitution samples solutions – Sample solutions reconstituted by using different diluents and diluted further to get final concentration 0.8 mg/ml.

**B. CHROMATOGRAPHIC CONDITIONS**

- **Column**: Nova pack, C-18 150 mm x 3.9mm, 4µ, Make: Waters
- **Column oven temperature**: 25°C
- **Sample compartment temperature**: 10°C
- **Flow rate**: 1.0 ml/min
- **Injection volume**: 10µl
- **Column temperature**: Ambient
- **Wavelength**: 235nm
- **Runtime**: 70 min
Gradient program:

<table>
<thead>
<tr>
<th>Time in mins</th>
<th>Mobile phase A (%)</th>
<th>Mobile phase B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>80</td>
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<td>16</td>
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<td>51</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>56</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>70</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

**Injections**

Separately injected 10 µL of blank (mobile phase), resolution solution, standard solution and sample solutions into the Waters HPLC and analysed the peak areas of Cefotaxime and its impurities using the Empower software II.

**System suitability**

Resolution between Cefotaxime and impurity-B from resolution solution was verified. Tailing factor Cefotaxime peak was verified from standard solution.
4.2 MATERIALS USED

A. EQUIPMENTS and SOFTWARE

Waters high pressure liquid chromatographic (HPLC) system was used bearing the configuration by equipped with pump (Waters 2695 separators module), auto sampler, thermo stated column compartment and detector (Waters 2487 dual wavelength absorbance detector) controlled by ‘Empower II’ software.

Agilent 1100 series high pressure liquid chromatographic (HPLC) system was also used bearing the configuration by equipped with pump, auto sampler, thermo stated column compartment and multi wavelength detector (MWD) controlled by the software ‘Chemstation’.

HPLC equipped with PDA (Waters 2996 Photodiode Array Detector) detector was used for measuring the peak purity during the forced degradation. Peak purity measured using the software Empower II software, where it measured purity angle and purity threshold. Peak purity claims as passed (no flag) if purity angle found less than purity threshold and claims as fails (flag exits) if purity angle found greater than purity threshold.

Millipore water purification system was used to collect purified water.

Sartorius analytical balance – model CP225D was used for weighing the materials

‘ChemDraw Ultra 8.0 - Chemical structure and Drawing standard’ software used to draw the chemical structures.

IR Spectrophotometer system – Model ‘IR Prestige – 21’ and Make – Shimadzu was used for identification analysis.
Materials & Methods

HPLC Columns

1. Hypersil, BDS C18, 150 x 4.6mm, 5µ, Make: Thermo
2. Symmetry ODS, 150mm X 4.6mm, 5µ, Make: Waters
3. Nova pack, C-18 150 mm x 3.9mm, 5µ, Make: Waters

B. MATERIALS

Samples and impurities

1. Ceftriaxone sodium working standard – Gift sample received from Kreszent Pharma, B.No – WS/09 with potency – 91.8% on as basis as Ceftriaxone.
2. Cefotaxime sodium working standard - Gift sample received from Kreszent Pharma, B.No – WS/30C-03 with potency – 91.53% on as basis as Cefotaxime.
3. Ceftriaxone sodium for injection 2.0g – Gift samples received from Kreszent Pharma.
4. Ceftriaxone sodium for injection 250mg (Mahacef 250) – Commercially available samples of manufacturer Mankind Pharma Ltd.
5. Ceftriaxone market available samples used -

<table>
<thead>
<tr>
<th>Sample No</th>
<th>I</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Product</td>
<td>Ceftriaxone Sodium for</td>
<td>Ceftriaxone Sodium for</td>
<td>Ceftriaxone Sodium for</td>
</tr>
<tr>
<td></td>
<td>injection 1g</td>
<td>injection 500mg</td>
<td>injection 1g</td>
</tr>
<tr>
<td>B.No</td>
<td>16G041</td>
<td>WZS9005</td>
<td>16G089</td>
</tr>
<tr>
<td>Brand Name</td>
<td>Monocef 1gm</td>
<td>Powercef 500mg</td>
<td>Monocef 1gm</td>
</tr>
<tr>
<td>Mfg.</td>
<td>Aristo Pharmaceuticals</td>
<td>Wockhardt Labs</td>
<td>Aristo Pharmaceuticals</td>
</tr>
</tbody>
</table>

6. Cefotaxime sodium for injection 2.0g - Gift samples received from Kreszent Pharma.
## Materials & Methods

### 7. Ceftriaxone impurities - Gift samples received from Kreszent Pharma

<table>
<thead>
<tr>
<th>Name of Impurity</th>
<th>Chemical Name of the Impurity</th>
<th>Batch no</th>
<th>Purity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Impurity-B</td>
<td>((5aR,6R)-6-[[2(Z)-(2-aminothiazol-4-yl)(methoxyimino)acetyl]amino]-5a,6-dihydro-3H,7H-azeto[2,1-b]furo[3,4-d][1,3]thiazine-1,7(4H)-dione)</td>
<td>WS/024</td>
<td>99.3</td>
</tr>
<tr>
<td>Impurity-C</td>
<td>7-aminocephalosporinc acid</td>
<td>WS/025</td>
<td>86.1</td>
</tr>
<tr>
<td>Impurity-D</td>
<td>2-methyl-3-sulphanyl-1,2-dihydro-1,2,4-triazine-5,6-dione</td>
<td>WS/026</td>
<td>96.4</td>
</tr>
<tr>
<td>Impurity-E</td>
<td>((6R,7R)-7-amino-3-[[2-methyl-5,6-dioxo-1,2,5,6-tetrahydro-1,2,4-triazin-3-yl]sulphanyl]methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid)</td>
<td>WS/026</td>
<td>99.2</td>
</tr>
<tr>
<td>Impurity-A</td>
<td>((6R,7R)-7-[[2(E)-(2-aminothiazol-4-yl)(methoxyimino)acetyl]amino]-3-[[2-methyl-5,6-dioxo-1,2,5,6-tetrahydro-1,2,4-triazin-3-yl]sulphanyl]methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid ((E)-isomer))</td>
<td>WS/029</td>
<td>93.5</td>
</tr>
</tbody>
</table>
8. Cefotaxime impurities - Gift samples received from Kreszent Pharma

<table>
<thead>
<tr>
<th>Name of Impurity</th>
<th>Chemical Name of the Impurity</th>
<th>Batch no</th>
<th>Purity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Impurity D</td>
<td>(6R,7R)-3-[(acetyloxy)methyl]-7-[[2E]-2-(2-aminothiazol-4-yl)-2-(methoxyimino)acetyl]amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (E-cefotaxime)</td>
<td>WS/031</td>
<td>91.53</td>
</tr>
<tr>
<td>Impurity E</td>
<td>(5aR,6R)-6-[[2Z]-2-(2-aminothiazol-4-yl)-2-(methoxyimino)acetyl]amino]-5a,6-dihydro-3H,7H-azeto[2,1-b]furo[3,4-d][1,3]thiazine-1,7(4H)-dione (deacetylcefotaxime lactone)</td>
<td>WS/032</td>
<td>79.2</td>
</tr>
<tr>
<td>Impurity B</td>
<td>(6R,7R)-7-[[2Z]-2-(2-aminothiazol-4-yl)-2-(methoxyimino)acetyl]amino]-3-(hydroxymethyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (deacetylcefotaxime)</td>
<td>WS/033</td>
<td>96.43</td>
</tr>
<tr>
<td>Impurity A</td>
<td>(6R,7R)-7-[[2Z]-2-(2-aminothiazol-4-yl)-2-(methoxyimino)acetyl]amino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (deacetoxycefotaxime)</td>
<td>WS/034</td>
<td>97.12</td>
</tr>
<tr>
<td>Impurity C</td>
<td>(6R,7R)-3-[(acetyloxy)methyl]-7-[[2Z]-2-[2-(formylamino)thiazol-4-yl]-2-(methoxyimino)acetyl]amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (N-formylectaxime)</td>
<td>WS/036</td>
<td>91.23</td>
</tr>
</tbody>
</table>
Chemicals

1. Disodium Hydrogen Orthophosphate anhydrous (Na$_2$HPO$_4$)
2. Tetraheptyl ammonium bromide
3. Sodium hydroxide
4. Conc. hydrochloric acid
5. Glacial Acetic acid
6. Ortho phosphoric acid (80% pure)
7. Acetonitrile
8. Methanol
9. Hydrogen peroxide
10. Potassium dihydrogen orthophosphate anhydrous (KH$_2$PO$_4$)
11. Iso Propyl Alcohol
12. Triethyl Amine
13. Ethylene Chloride
14. Sodium 2-ethyl haxanoate
15. Active Carbon charcoal.
16. Ether

The above chemicals are of analytical grade and obtained from Merck. These were procured form commercial source and purified water was obtained using Millipore water purification system.

17. ATMA - (Z)-(2-Aminothiazol-4-yl)-2-(methoxyimino) acetic acid
18. 7-ACA – 7-Amino Cephalosporinic acid.

The above chemicals are procured from commercial source of Sigma Aldrich.

Materials & Methods

**Intramuscular and Intravenous diluents**

1. Sterile water for injection - (Mfg: Core healthcare limited, batch no.: 501533 and 5027795)
2. 0.9% sodium chloride solution - (Mfg: Claris Life sciences limited, batch no. 3.11.6565 and 1703346)
3. 10% dextrose solution - (Mfg: Albert David limited , batch no.: P4B971 and (Mfg: Claris Life Sciences Limited, batch no. 4.02.7985))
4. 5% Dextrose solution - (Mfg: Albert David limited, batch no.: 5AC97 and 5AC72)
5. Sodium lactate solution - (Mfg: Baxter (India) Pvt. ltd, batch no: 957654)
6. 10% Invert sugar - (Mfg: Raptakos, Brett and co. Ltd, batch no: TW 4389 B)
7. 5% Sodium bi carbonate - (Mfg: Superb Drugs Pvt. Ltd., batch no: S93)
8. Free amine III - (Mfg: Claris Life science Limited, batch no.: 0-09-6174)
9. Metronidazole HCl - (Mfg: JB Chemicals and Pharmaceuticals Ltd, batch no: D8876 and D6245)
10. 10% Invert sugar - (Mfg: Raptakos, Brett and Co. Ltd, B. No.: TW 4561A)
11. Lactated ringers injection - (Mfg: Claris Life Sciences Limited, B. No.: 40.2998)
12. 8% TRAVASOL - (amino acid) injection without electrolyte (Mfg: Albert David Limited, B. No.: SN5A19).
13. 2% Lignocaine - (Mfg: Astrazeneca Pharma India Limited, B. No.: XYZD089).

The above diluents are procured from commercial source.