Chapter -3
Material and Methods
4.1 LOPINAVIR & RITONAVIR SOFT GELATIN CAPSULES.

Structure of Lopinavir:
A) Structure of Ritonavir:

Chemical name of Lopinavir:

Mole. Formula of Lopinavir: $\text{C}_{37}\text{H}_{48}\text{N}_{4}\text{O}_{5}$
Mole. Wt-of Lopinavi: 628.81.
Mole. Formula of Ritonavir: $\text{C}_{37}\text{H}_{48}\text{N}_{6}\text{O}_{5}\text{S}_{2}$
Mole. Wt-of Ritonavir: 720.95

Limit:
% Assay limit for Lopinavir is not less than 90.0% and not more 110.0% of label claim.
% Assay limit for Ritonavir is not less than 90.0% and not more 110.0% of label claim
4.1.1 SCOPE:
In soft gelatin capsules assay HPLC method for analysis of Lopinavir as well as Ritonavir is developed and validated. This Method Applicable for Assay and Content Uniformity in combination drug of Lopinavir & Ritonavir Soft gel form. 
Related Substances method also developed for combination drug of Lopinavir & Ritonavir Soft gel form.

**Instruments to be used:**
High performance Liquid Chromatograph. (UV / Photo Diode Array detector).

**Column to be used:**
1. C-18 , 50 mili metre x 4.6 mili metre, 3.5 µm. Zorbax SB
2. C-18, 250 mili metre x 4.6 mili metre, 5 µm. Agilent, Zorbax SB
3. ACE C4, 15 centi m x4.6 mili-meter3 µm.

**Standard to be used**

<table>
<thead>
<tr>
<th>Name</th>
<th>Chromatographic Purity (in %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lopinavir</td>
<td>100.77 (Anhydrous Basis)</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>100.00 (As is Basis)</td>
</tr>
</tbody>
</table>

**Sample to be used:**
Lopinavir & Ritonavir Soft gelatin Capsules.
Chemicals and Reagent:

Table 2: Chemicals and Reagent:

<table>
<thead>
<tr>
<th>Name of Chemicals</th>
<th>Make</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dihydrogen Potassium orthophosphate</td>
<td>Rankem</td>
</tr>
<tr>
<td>ACN-(Acetonitrile)</td>
<td>Rankem</td>
</tr>
<tr>
<td>Methanol</td>
<td>Rankem</td>
</tr>
<tr>
<td>Orthophosphoric acid</td>
<td>Rankem</td>
</tr>
<tr>
<td>Tetrahydrofuran</td>
<td>Rankem</td>
</tr>
<tr>
<td>N-Butanol</td>
<td>Rankem</td>
</tr>
<tr>
<td>Hydrochloric acid</td>
<td>Merck</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>Qualigen</td>
</tr>
<tr>
<td>Sodium Hydroxide</td>
<td>Merck</td>
</tr>
</tbody>
</table>
4.1.2 Soft gel capsules of Lopinavir & Ritonavir combination formulation – Assay Method.

Developed Method for Lopinavir- Ritonavir Assay and content Uniformity:

**System Controlling Parameter**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name of system</td>
<td>HPLC</td>
</tr>
<tr>
<td>Column used</td>
<td>C-18, 50 millimeter x 4.6 millimeter, 3.5 µm. Zorbax SB</td>
</tr>
<tr>
<td>System flow-rate</td>
<td>1.0 milliliter / minute</td>
</tr>
<tr>
<td>Absorption Wavelength</td>
<td>210 Nano-meter</td>
</tr>
<tr>
<td>Temperatures of Column oven</td>
<td>25°C</td>
</tr>
<tr>
<td>Volume at time of injection</td>
<td>10 µL</td>
</tr>
<tr>
<td>Run time of injection</td>
<td>15 Minutes</td>
</tr>
<tr>
<td>Mode type For Analysis</td>
<td>Isocratic.</td>
</tr>
</tbody>
</table>

**Buffer Preparation:**

Dissolve 6.8 g Dihydrogen Potassium phosphate in to 1 liter water. Make pH for this buffer Sample to 3.00 ± 0.05 Unit with (O-phosphoric Acid) OPA. Filter it from Zero point Forty five (0.4 micro meters) um nylone filter.

**Making of Dilute Orthophosphoric acid Sample:**

Take 200 milli liter of flask to it added 20 milli liter of phosphoric acid (OPA) and mixed water to ring mark of flask.

**Making of Solvent Mixture Sample:**

Homogeniously mixed 800 milli liter of acetonitrile and 200 milli liter of methanol to make solvent mixture sample.

**Preparation of Mobile-Phase:**

The Mixture of filtered degassed buffer and Solvent Mixture in proportion of 450:550
Diluent Sample:

I. Methanol
II. Mobile Phase

Making of Lopinavir Stock Sample:
Weigh accurately about 35.01 mg of Lopinavir standard in 50 milli liter of flask to it add 35 milli liter of Methanol dissolve and make up it to volume (ring mark) of flask with Methyl alcohol (Methanol).

Making of Ritonavir Stock Sample:
Weigh accurately about 35.10 mg of Ritonavir standard in 200 milli liter of flask to it add 150 milli liter of Methanol dissolve and make up it to volume (ring mark) of flask with Methyl alcohol (Methanol).

Making of Standard Sample (Reference Sample):
Added 5 ml each of stock Sample to 50.0 mili liter flask (Volumetric) and diluted to 50 mili liter to volume (ring mark) of flask using diluent i.e. Mobile-phase.

Test Sample Preparation for Assay:
Mix Content of 20 Capsules. Transfer an accurately weigh quantity of content from capsule Equal to Lopinavir about 166 mg and Ritonavir about 41 mg in 250 milli liter of flask. Add 170 ml of Methanol, Sonicate it through occasional Slight Shaking for 25 Minutes diluted to ring level with help of Methyl alcohol (Solvent-Methanol) and mixed well. By filtration of Sample from Zero point Forty five (0.4 micro meter) Micron nylon size needle-syringe filter by removing first 3 ml solution and Again dilute to 50 milliliter volumetric flask with help of mobile phase having 5 milliliter of this Sample (stock Sample).

Test Sample Preparation for Content Uniformity:
Transfer one capsule in 200 milli liter of flask. then by adding about 35 mili liter diluent- mobile phase give wave sonication of 15 minute to dispersed Shell, then add
100 ml Methanol Sonicate it through occasional Slight Shaking for 30 Minutes make up to flask ring level with help of Methyl alcohol (Solvent-Methanol) and mixed well. By filtration of Sample from Zero point Forty five (0.4 micro meter) Micron nylon size needle-syringe filter by removing first 3 ml solution and Again dilute to 50 milliliter volumetric flask with help of mobile phase having 5 milliliter of this Sample (stock Sample).

Check Points for capability of instrument and the limit for respective are as,
The % RSD for five injection of Standard NMT 2.0%.
The factor for tailing for the Lopinavir & Ritonavir peak NMT 2.0.
The theoretical plates would more than 2000 for the Lopinavir & Ritonavir.

**Injection scheme:**

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Name of injection to be injected</th>
<th>Replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Blank solution</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Standard Sample (Reference Sample)</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>Assay Sample solution-I</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>Assay sample solution-II</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>Suitability check standard</td>
<td>1</td>
</tr>
</tbody>
</table>

By processing all chromatogram to given absorption wavelength measure percentage of both content from combination samples.

**Calculations:**

**Percentage Assay in capsule for Lopinavir =

\[
Y = \left( \frac{A \times C \times 5 \times 250.0 \times 50.0 \times (100-w) \times P1}{B \times 50 \times 50 \times D \times 5.0 \times 100.0 \times 100.0} \right) \times 100
\]
Content of Lopinavir = -------------------------------
                   L.A

Where,
A : Area for Lopinavir in to assay sample solution
B : Mean area for Lopinavir in Reference Sample.
C : Wt of Lopinavir standard used during analysis.
D : sample Wt at time of sample preparation (mili-gram)
P : Label claim of drug calculated
Net Fill : Net liquid filled contain (Mili-gram)
P : standard percent purity of respective drug (Lopinavir).
L.A. : Labelled Amount.

% Assay of Ritonavir =

\[
Z = \frac{A \times C}{B} \times \frac{50}{50.0} \times \frac{250.0}{50.0} \times \frac{50.0}{D} \times \frac{5.0}{5.0} \times \frac{100.0}{100.0} \times 100
\]

Content of Ritonavir = -------------------------------
                   L.A.

Where,
A : Area for Ritonavir in to assay sample solution
B : Mean area for Ritonavir in Reference Sample.
C : Wt of Ritonavir standard used during analysis.
D : sample Wt at time of sample preparation (mili-gram)
P : Label claim of drug calculated
Net Fill : Net liquid filled contain (Mili-gram)
P : standard percent purity of respective drug (Ritonavir).
L.A. : Labelled Amount.
4.1.2 ANALYTICAL METHOD FOR RELATED SUBSTANCES OF LOPINAVIR IN LOPINAVIR & RITONAVIR SOFT GELATIN CAPSULES.

Related substances (Impurity determination)

Chromatographic Conditions:

System Controlling Parameter

Name of system : HPLC
Used Column : SB C-18, 250 mili miter x 4.6 mili miter x 5 µm. Zorbax Agilent.
System flow-rate : 1.5 milliliter / minute
Absorption Wavelength : 210 Nano-meter
Temperatures of Column oven : 30°C
Volume at time of injection : 20 µL
Run time of injection : 90 Minutes
Mode type For Analysis : Gradient

Gradient Programme:

Gradient Programme for Related Substances

<table>
<thead>
<tr>
<th>Time in Minute</th>
<th>% - A (Phase)</th>
<th>% - B (Phase)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.010</td>
<td>100.00</td>
<td>0.00</td>
</tr>
<tr>
<td>80.00</td>
<td>100.00</td>
<td>0.00</td>
</tr>
<tr>
<td>90.00</td>
<td>0.00</td>
<td>100.00</td>
</tr>
<tr>
<td>120.00</td>
<td>0.00</td>
<td>100.00</td>
</tr>
<tr>
<td>130.00</td>
<td>100.00</td>
<td>0.00</td>
</tr>
<tr>
<td>140.00</td>
<td>100.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Buffer Preparation pH 4.5:

Dissolved 1.400 g Potassium phosphate (Dihydrogen) to 1 liter Purified water. Make pH for this buffer Sample to 4.50 ± 0.05 Unit with (O-phosphoric Acid) OPA. Filtration done from Zero point Forty five (0.4 micro meters) um nylon filters.
Making of Dilute Orthophosphoric acid Sample:
Take 200 mili liter of flask to it added 20 mili liter of phosphoric acid (OPA) and mixed water to ring mark of flask.

Making of Mobile Phase - (A):
Used completely filtered Homogenous Mixture buffer pH 4.5 and ACN (Acetonitrile) has proportion of 55:45.

Making of Mobile Phase - (B):
Used completely filtered Homogenous Mixtured -buffer pH 4.5 and ACN (Acetonitrile) has proportion of 80:20.

Diluent Sample:
Prepared Homogenous Mixtured buffer pH 4.5 and ACN (Acetonitrile)) has proportion of 20:80

Making of Lopinavir Standard Sample:
Weighed and transferred 10.00 mg of Lopinavir standard in 100 milli liter of flask to it added 70 milli liter diluent-solution dissolved through sonication and make up it to volume (ring mark) of flask with diluent. Again dilute 1 milliliter of respective solution to 100 milli liter of flask through help of diluent-solution.

Making of Placebo Sample:
Weighed and transferred 0.240 g of placebo and 10.00 mg of Ritonavir standard in 50 milli liter of flask to it added 30 milli liter diluent-solution dissolved through sonication and make up it to volume (ring-mark) through help of diluent-solution.

Test Sample Preparation for:
Mixed Content of 20 Capsules. Transferred weight equal to 50 mili-gram lopinavir drug in 50 milli liter of flask. Added 30 ml of diluent, Sonicate it through occasional Slight Shaking for 10 Minutes in ice cold water, cool to Ambient temperature (Room temperature), make up to flask ring level with help of diluent diluent-solution and homogeniously mixed.
Check Points for capability of instrument and the limit for respective are as,

1. For standard solution preparation-Asymmetry (Tailing) for both (Lopinavir & Ritonavir) not more than 2.00
2. For standard solution preparation - Theoretical plates for both drug (Lopinavir & Ritonavir) not less than 2000.
3. % RSD would NMT 5.0. for both peak (Lopinavir & Ritonavir)

Injection scheme for Related Substances

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Name of injection to be injected</th>
<th>Replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Blank- solution (Diluent)</td>
<td>Replicate- 1</td>
</tr>
<tr>
<td>ii</td>
<td>Standard - Sample preparation</td>
<td>Replicate- 6</td>
</tr>
<tr>
<td>iii</td>
<td>Placebo - Sample preparation</td>
<td>Replicate- 1</td>
</tr>
<tr>
<td>iv</td>
<td>Sample -solution preparation</td>
<td>Replicate- 1</td>
</tr>
<tr>
<td>v</td>
<td>Suitability check standard</td>
<td>Replicate- 1</td>
</tr>
</tbody>
</table>

By processing all chromatogram to given absorption wavelength measure percentage related substances for Lopinavir from combination samples.

Calculations:

**Determination of Impurity (Lopinavir) (%) =**

\[
\frac{A \times C \times 1.0 \times 50.0 \times (100-W) \times P \times 100.0}{B \times 100.0 \times 100.0 \times D \times 100.0 \times 100.0 \times \text{LA}}
\]

Where,

A : Observed in unknown peak for sample.
B : Observed Mean peak area from replicates for Lopinavir standard
C : Standard net wt taken in mili-gram.
D : Sample net wt taken in mili-gram.
W : % Moisture Content observed in Lopinavir.
Net Fill : Average wt-of capsules filled (mili-gram)
P : Purity of Lopinavir Standard.
L.A. : Labelled Amount.
4.1.3 DEVELOPED LIQUID CHROMATOGRAPHIC METHOD FOR DETERMINATION RITONAVIR IMPURITIES IN COMBINATION CAPSULES OF LOPINAVIR WITH RITONAVIR.

Ritonavir Related substances (Impurity Determination):
System Controlling Parameter
Name of system : HPLC
Used Column : 15 centimeter x4.6 millimeter μm, ACE C4.
System flow-rate : 1.2 milliliter / minute
Absorption Wavelength : 240 Nano-meter
Temperatures of Column oven : 55°C
Volume at time of injection : 50 μL
Run time of injection : 155 Minutes
Mode type For Analysis : Gradient

Gradient Programme:

Gradient Programme for Related Substances

<table>
<thead>
<tr>
<th>Time in Minute</th>
<th>% - A (Phase)</th>
<th>% - B (Phase)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>100.00</td>
<td>0.00</td>
</tr>
<tr>
<td>60.00</td>
<td>100.00</td>
<td>0.00</td>
</tr>
<tr>
<td>120.00</td>
<td>0.00</td>
<td>100.00</td>
</tr>
<tr>
<td>120.10</td>
<td>100.00</td>
<td>0.00</td>
</tr>
<tr>
<td>155.0</td>
<td>100.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Buffer Preparation:
Dissolved 4.120 g Dihydrogen Potassium phosphate in to 1 liter water. Filter it from Zero point Forty five (0.4 micro meters) um nylon filters.

Making of Mobile Phase A:
Used completely filtered Homogenous Mixtured -buffer, ACN (Acetonitrile), Tetrahydrofuran, n-Butanol in proportion of 70:19:8:5.
Making of Mobile Phase -(B) :
Used completely filtered Homogenous Mixtured-buffer, ACN (Acetonitrile), Tetrahydrofuran, n-Butanol in proportion of 40:45:10:5.

Diluent Sample:
Prepared Homogenous Mixtured -buffer and ACN (Acetonitrile) has proportion of 40:60

Stock solution preparation for Ritonavir working standard:
Weighed 25.10 mg of Ritonavir working standard to 50.0 milliliter of flask to it added 30 milliliter diluents solution dissolved it through wave sonicater and diluted this flask to its mark level through help of diluent solution.

Stock solution preparation for Impurities mixture:
Weighed 15.05 mg of Impurity E, 10 mg Impurity F and impurity I, 7.5 mg of impurity L and impurity C in 100 milliliter of flask to it add 70 milliliter diluent and dissolved through wave sonicater, added diluents to mark (ring mark) of flask with diluents
Solution mix homoginiously.

Preparation for Ritonavir working standard:
Diluted 2.0 milliliter each of Ritonavir stock and Impurities stock to 200 milliliter of volumetric flask. Added diluents to final volume of 200 milliliter flask, homoginiously mix it.

Making of Placebo Sample:
Weighed and transferred 0.475 g of placebo and 98.50 mg of Lopinavir standard in 50 milli liter of flask to it add 30 milliliter of diluent dissolved through wave solicited diluted to volume (ring mark) of flask with help of diluents solution.

Test Sample Preparation: -
Mixed Content of 20 Capsules, Transferred weighed content of capsules equal to 25.0 milligram of Ritonavir in 50.0 milliliter of flask. Added 30 ml of diluent, Sonicate it through occasional Slight Shaking for 10 Minutes in ice cold water, cool
to Ambient temperature (Room temperature), make up to flask ring level with help of diluent and mix..

Check Points for capability of instrument and the limit for respective are as,

1. For standard solution preparation-Asymmetry (Tailing) for both (Lopinavir & Ritonavir) not more than 2.00
2. For standard solution preparation - Theoretical plates for both drug (Lopinavir & Ritonavir) not less than 2000.
3. % RSD would NMT 5.0. for both peak (Lopinavir & Ritonavir)

**Injection scheme for Related Substances**

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Name of injection to be injected</th>
<th>Replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Blank- solution (Diluent)</td>
<td>Replicate- 1</td>
</tr>
<tr>
<td>ii</td>
<td>Standard - Sample preparation</td>
<td>Replicate- 6</td>
</tr>
<tr>
<td>iii</td>
<td>Placebo - Sample preparation</td>
<td>Replicate- 1</td>
</tr>
<tr>
<td>iv</td>
<td>Sample -solution preparation</td>
<td>Replicate- 1</td>
</tr>
<tr>
<td>v</td>
<td>Suitability check standard</td>
<td>Replicate- 1</td>
</tr>
</tbody>
</table>

By processing all chromatogram to given absorption wavelength measure percentage related substances for Ritonavir from combination samples.

Calculations:

**Determination of Impurity (Lopinavir) (%)** =

\[
\frac{A \times C \times 1 \times 50 \times P \times 100}{\text{Net Filled}}
\]

\[
\text{Net Filled} = \frac{B \times 100 \times D \times 100 \times S \times LA}{\text{Net Filled}}
\]
Where,

A : Observed in unknown peak for sample.
B : Observed Mean peak area from replicates for Ritonavir standard
C : Standard net wt taken in milli-gram.
D : Sample net wt taken in milli-gram.
Net Fill : Average wt-of capsules filled (milli-gram)
P : Purity of Lopinavir Standard.
L.A. : Labelled Amount.
4.1.4 Forced degradation study is carried out on optimized method. only degradation product (Sulphoxide impurity) is monitored.

System Controlling Parameter

<table>
<thead>
<tr>
<th>Name of system</th>
<th>: HPLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Used Column</td>
<td>: C18, 150 x 4.6mm, 5 µ, (Zorbax SB)</td>
</tr>
<tr>
<td>System flow-rate</td>
<td>: 1.2 milliliter / minute</td>
</tr>
<tr>
<td>Absorption Wavelength</td>
<td>: 225 Nano-meter</td>
</tr>
<tr>
<td>Temperatures of Column oven</td>
<td>: 30°C</td>
</tr>
<tr>
<td>Volume at time of injection</td>
<td>: 20 µL</td>
</tr>
<tr>
<td>Run time of injection</td>
<td>: 40 Minutes</td>
</tr>
<tr>
<td>Used Diluent</td>
<td>: Purified Water and Acetonitrile (25 : 75)</td>
</tr>
<tr>
<td>Mode type For Analysis</td>
<td>: Gradient</td>
</tr>
</tbody>
</table>

Gradient:

<table>
<thead>
<tr>
<th>Time</th>
<th>Phase A %</th>
<th>Phase B %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>3.0</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>7.0</td>
<td>39</td>
<td>61</td>
</tr>
<tr>
<td>20.0</td>
<td>39</td>
<td>61</td>
</tr>
<tr>
<td>25.0</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>32.0</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>34.0</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>40.0</td>
<td>60</td>
<td>40</td>
</tr>
</tbody>
</table>

Preparation of Buffer:

Transferred 1.362 gram Potassium Phosphate (monohydrate) in 1000 milliliter of purified water. Added 1 milliliter of TEA (Triethylamine) made the pH of buffer to 6.05 with dilute OPA (Orthophosphoric acid) degassed it by filtered through 0.45µ filter.

Mobile Phase A: Buffer directly used as phase for Port -A

Mobile Phase B: In Port –B used 100% acetonitrile.
<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Condition</th>
<th>Degrading agents / Conditions</th>
<th>Exposure period</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Acid degradation</td>
<td>1N HCl (aqueous)</td>
<td>For 2 hours RT</td>
</tr>
<tr>
<td>2</td>
<td>Base degradation</td>
<td>1N NaOH (aqueous)</td>
<td>For 2 hours RT</td>
</tr>
<tr>
<td>3</td>
<td>Peroxide dégradation</td>
<td>30% H₂O₂ (aqueous)</td>
<td>For 0 Minutes</td>
</tr>
<tr>
<td>4</td>
<td>Heat degradation (Solid state)</td>
<td>80°C</td>
<td>For 6 hours</td>
</tr>
<tr>
<td>5</td>
<td>Heat degradation (Solution state)</td>
<td>60°C</td>
<td>For 2 hours</td>
</tr>
<tr>
<td>6</td>
<td>Humidity degradation</td>
<td>75% R.H.</td>
<td>For 24 hours</td>
</tr>
<tr>
<td>7</td>
<td>Photolytic degradation</td>
<td>----</td>
<td>1.2 Million lux hrs more than 200 watt hrs per square meter.</td>
</tr>
</tbody>
</table>

Table: 4. Peak purity data: Forced degradation study of Montelukast chewable Tablets

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Stress type</th>
<th>Degrading agents / condition</th>
<th>Exposure period</th>
<th>Peak purity criteria of Montelukast peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Untreated sample</td>
<td>-</td>
<td>-</td>
<td>Pass</td>
</tr>
<tr>
<td>2</td>
<td>Acid degradation</td>
<td>1N HCl (aqueous)</td>
<td>2 hours RT</td>
<td>Pass</td>
</tr>
<tr>
<td>3</td>
<td>Base degradation</td>
<td>1N NaOH (aqueous)</td>
<td>2 hours RT</td>
<td>Pass</td>
</tr>
<tr>
<td>4</td>
<td>Peroxide dégradation</td>
<td>30% H₂O₂ (aqueous)</td>
<td>0 min</td>
<td>Pass</td>
</tr>
<tr>
<td>5</td>
<td>Heat degradation (Solid state)</td>
<td>80°C</td>
<td>6 hours</td>
<td>Pass</td>
</tr>
<tr>
<td>6</td>
<td>Heat degradation (Solution state)</td>
<td>60°C</td>
<td>2 hours</td>
<td>Pass</td>
</tr>
<tr>
<td>6</td>
<td>Humidity degradation</td>
<td>75% R.H.</td>
<td>For 24 hours</td>
<td>Pass</td>
</tr>
<tr>
<td>7</td>
<td>Photolytic degradation</td>
<td>----</td>
<td>1.2 Million lux hrs more than 200 watt hrs per square meter.</td>
<td>Pass</td>
</tr>
</tbody>
</table>
Remark:
A] The degrading peaks of Montelukast are found well resolved from the Montelukast peak in the degradation samples at all degradation conditions.
B] The peak purity of Montelukast passes at all degradation conditions.
C] The degradants peaks of excipient blend do not interfere the peak of Montelukast at each condition of degradation.
D] Based on the above observations it is concluded that the method is selective, stability indicating, specific and can be use for analysis.
Figure 1.25: Diluent Blank:
Figure 1.26: System suitability solution:
Figure 1.27: Placebo- Untreated:
Figure 1.28: Placebo Oxidation Degradation:
Figure 1.29: Placebo- Acid Degradation:

Sample ID: Placebo_1 N HCL_2 Hrs_RT
Figure 1.30: Placebo- Base Degradation:

Sample ID: Placebo_1 N NaOH_2 Hrs RT
Figure 1.40: Placebo- Heat Degradation (Solution state):
Figure 1.41: Placebo- Heat Degradation (Solid state):
Figure 1.42: Placebo-Humidity:
Figure 1.43: Placebo- Photolytic Degradation:
Figure 1.44: Tablet Sample- Untreated:
Figure 1.45: Tablet Sample - Oxidation degradation:
Figure 1.46: Tablet Sample - Acid degradation:

Sample ID: TAB SAMPLE 1 N HCL 2 Hrs RT

- Sulfoxide 1: 7.717, 6.473
- Sulfoxide 2: 13.310
- Montaluka est: 15.362
- Methyl ether impurity: 26.428
- 28.886
- 32.141
Figure 1.47: Tablet Sample- Base degradation:

Sample ID: TAB SAMPLE_1 N NaOH_2 Hrs_RT
Figure 1.48: Tablet Sample - Heat degradation (Solution state):

Sample_ID: TAB SAMPLE Heat(Soln state) 2 Hrs 60°C
Figure 1.49: Tablet Sample - Heat degradation (Solution state):

Sample ID: TAB SAMPLE_Heat(Solid state)_6 Hrs_80°C
Figure 1.50: Tablet Sample- Heat degradation (Solid state):
Figure 1.51: Tablet Sample- Humidity degradation:
Figure 1.52: Tablet Sample- Photolytic degradation:

Sample_ID: TAB SAMPLE_Photolytic

[Graph showing chromatogram with peaks labeled as Sulphoxide 1, Sulphoxide 2, Montelukast, Methyl ether impurity, and Styrene impurity]
4.1.5 ANALYTICAL METHOD FOR ASSAY OF MONTAIR TABLETS.

Montelukast is leukotriene receptor which very used full to treat asthmatic adults and small children. Mostly this is useful to treat children below six years old. Now a day most of new born are affected from asthma. Hence this drug plays major role in the asthmatic treatment.

Structure:

![Structure of Montelukast](image)

Figure 2.3: MONTELUKAST Na

Table: 5. Details of molecule for Montelukast

<table>
<thead>
<tr>
<th>Details of molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mole. Formula plane Montelukast</td>
</tr>
<tr>
<td>Mole. Wt. plane Montelukast</td>
</tr>
<tr>
<td>Mole. formula with sodium</td>
</tr>
<tr>
<td>Mole. Wt with sodium</td>
</tr>
</tbody>
</table>

Limit:
The limits for assay of Montelukast in Montelukast Sodium Chewable Tablets 5 mg are not less than 90.0% and NMT 110.0%.

Instruments to be used:
Waters system: Auto sampler with PDA
Detector: PDA
Column to be used: C-18, 15 centimetre X 4.6 millimeter X 5 µ Waters

Sample to be used:
Montelukast Sodium Tablets-Market Sample

Excipient Blend to be used:
Excipient blend will be prepared in house as per the manufacturing formula except the active pharmaceutical ingredient.

Montelukast: Analytical method

<table>
<thead>
<tr>
<th>System Controlling Parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name of system</td>
</tr>
<tr>
<td>Used Column</td>
</tr>
<tr>
<td>System flow-rate</td>
</tr>
<tr>
<td>Absorption Wavelength</td>
</tr>
<tr>
<td>Temperature of Column oven</td>
</tr>
<tr>
<td>Volume at time of injection</td>
</tr>
<tr>
<td>Run time of injection</td>
</tr>
<tr>
<td>Mode type For Analysis</td>
</tr>
</tbody>
</table>

Gradient Programme

<table>
<thead>
<tr>
<th>Time in Min</th>
<th>Mobile Phase A %</th>
<th>Mobile Phase B %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>35</td>
<td>65</td>
</tr>
<tr>
<td>10</td>
<td>35</td>
<td>65</td>
</tr>
<tr>
<td>12</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>16</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>17</td>
<td>35</td>
<td>65</td>
</tr>
<tr>
<td>25</td>
<td>35</td>
<td>65</td>
</tr>
</tbody>
</table>

Buffer Preparation:
Transferred 1.362 gram Potassium Phosphate (monohydrate) in 1000 milliliter of purified water. Added 1 milliliter of TEA (Triethylamine) made the pH of buffer to
6.05 with dilute OPA (Orthophosphoric acid) degassed. Filter it from Zero point Forty five (0.4 micro meters) um nylon filter.

**Phase A :**
Above buffer used as phase-A in gradient system for port-A.

**Phase B :**
Plane Acetonitrile used as phase-B in gradient system for port-B.

**Diluents for Dilution of sample:**
Mixed 500 milliliter of purified water with 1500 milliliter ACN (Acetonitrile)

Making of Montelukast sodium Standard Sample (Reference Sample):
In 50.0 milliliter of volumetric flask added 26.21 milligram of Montelukast Na standard to it added 40 milliliter of diluents sample, dissolved it through wave Sonicator, then dilute to mark of the flask through help of diluents. Further dilute 5.0 milliliter of this stock solution to 25 milliliter of flask.

**Test Sample Preparation:**
Take average weight of twenty tablets crushed it with very fine powder with help of mortar and pestle, transferred his powder equal to 10 milligram of Montelukast in 100 milliliter of volumetric flask. To this added 80 milliliter of diluent and give shaking to flask to disintegrate powder properly, sonicate through wave sonicator dilute to ring mark of flask and filter this Sample from Zero point Forty five (0.4 micro meter) Micron nylon size Syringe filter

**Check Points for capability of instrument and the limit for respective are as,**
The % RSD for five injection of Standard NMT 2.0%.
The factor for tailing for the Montelukast peak NMT 2.0.
The theoretical plates would more than 3000 for the Montelukast peak.

**Injection scheme:**

<table>
<thead>
<tr>
<th>Sr.</th>
<th>Samples series</th>
<th>No. of replicates</th>
</tr>
</thead>
</table>

42
<table>
<thead>
<tr>
<th>No.</th>
<th>Description</th>
<th>Replicate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Blank</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Standards</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>Tablets sample-I</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>Tablets Sample-II</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>Check Standard</td>
<td>1</td>
</tr>
</tbody>
</table>

**Calculations:**

(5 mg)

\[
\text{% Assay} = \frac{A}{B} \times \frac{C}{50} \times \frac{10}{50} \times \frac{100}{D} \times \frac{586.18}{608.17} \times \frac{\text{Avg. Wt.}}{\text{L.A.}} \times P
\]

Where,

- \( A \): Main peak observed area in tablet sample.
- \( B \): Average Main peak observed area in tablets standards.
- \( C \): Wt-of Montelukast Na Standard (milligram)
- \( D \): Wt-of tablets powder (milligram)
- 586.18: Molecular wt-of Montelukast.
- 608.17: Molecular wt-of Montelukast Sodium.
- \( \text{L.A.} \): Label claim.
- \( \text{Avg. Wt} \): Average wt-of tablets (milligram)
- \( P \): Purity of Standard.
5.1.0 ANALYTICAL METHOD VALIDATION FOR ASSAY OF LOPINAVIR & RITONAVIR SOFT GELATIN CAPSULES AND MONTAIR TABLETS

VALIDATION PARAMETERS

Parameters to be validated are as follows:

1. Specificity
   1.1 Selectivity
   1.2 Forced Degradation
2. Linearity and Range
3. Precision
4. Recovery ie.accuracy (% Recovery)
5. Filter Validation
6. Robustness
7. Stability of Analytical Sample
### Table 6: Acceptance Criteria for Assay of Lopinavir & Ritonavir Soft Gelatin Capsules.

<table>
<thead>
<tr>
<th>Validation parameter</th>
<th>Given</th>
<th>Range of study</th>
<th>Acceptance criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specificity</td>
<td>----</td>
<td>By injecting Diluent blank Sample, excipient blend Sample (placebo), known impurities, test Sample and test Sample spiked with known impurities.</td>
<td>The peaks due to Lopinavir &amp; Ritonavir Would be completely resolved from any other peak. The excipient blend, diluent blank and known Impurity Would not show any peak at the RT of the Lopinavir &amp; Ritonavir peak.</td>
</tr>
<tr>
<td>- Selectivity</td>
<td>----</td>
<td>The forced degradation studies would be performed using 1 N HCl, 1N NaOH, 30% H₂O₂. Also effect of temperature and photolysis would be checked.</td>
<td>The degradation Peak Would be completely resolved from the peak of Lopinavir &amp; Ritonavir. Degradation would Found any any one of the stress condition. Peak purity would passes for the peak due to Lopinavir &amp; Ritonavir</td>
</tr>
<tr>
<td>Specificity - Forced degradation study</td>
<td>----</td>
<td>50% to 150%</td>
<td>correlation co-efficient for this test would be equal or not Less than 0.99 Y intercept as obtain in linearity would be ± 2.0</td>
</tr>
<tr>
<td>Linearity and Range</td>
<td>----</td>
<td>----</td>
<td>% RSD peak areas for five injection of standard would NMT 2.0</td>
</tr>
<tr>
<td>System Precision</td>
<td>----</td>
<td>----</td>
<td>In this test % assay value calculate and % relative SD would not exceed 2.0 % value.</td>
</tr>
<tr>
<td>Validation parameter</td>
<td>Given</td>
<td>Range of study</td>
<td>Acceptance criteria</td>
</tr>
<tr>
<td>----------------------</td>
<td>-------</td>
<td>----------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>Recovery ie.accuracy (%Recovery)</td>
<td>----</td>
<td>At 50%, 100% &amp; 150% (for it Sample concentration to be consider)</td>
<td>Result found for this test would be in range of 98.00 % -102.00 %. (it shows accuracy of method) For all levels percentage RSD would not exceed than 2.00 %</td>
</tr>
<tr>
<td>Filter Validation</td>
<td>----</td>
<td>Unfiltered and filtered sample.</td>
<td>The difference between test Sample filtered and test Sample unfiltered would not be more than 2.0%</td>
</tr>
<tr>
<td>Robustness – phase flow change respective to actual flow of system (± 0.1 ml)</td>
<td>1.0 ml</td>
<td>0.9 ml and 1.1 ml</td>
<td>% RSD between results obtained with changed condition and six results of method precision would not be more than 2.0.</td>
</tr>
<tr>
<td>Robustness – change in pH - buffer (± 0.2 unit)</td>
<td>3.00</td>
<td>2.8 and 3.2</td>
<td>% RSD between results obtained with changed condition and six results of method precision would not be more than 2.0.</td>
</tr>
<tr>
<td>Stability of analytical Sample</td>
<td>At Room 2°C to 8°C</td>
<td>Evaluate the Sample stability for on hours Basis for test Sample and standard Sample (Reference Sample).</td>
<td>The Sample is considered stable, till the time point where the % RSD of the stored and initial prepared test and standard Sample (Reference Sample) is NMT 2.0.</td>
</tr>
</tbody>
</table>
5.1.2 ANALYTICAL METHOD VALIDATION PARAMETER AND ITS CRITERIA FOR ASSAY OF MONTAIR TABLETS.

Table 7: Established Acceptance Criteria for Assay of montair Tablets.

<table>
<thead>
<tr>
<th>Validation parameter</th>
<th>Specification</th>
<th>Range of study</th>
<th>Acceptance criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specificity</td>
<td>----</td>
<td>By injecting Diluent blank Sample, excipient blend Sample (placebo), known impurities, test Sample and test Sample spiked with known impurities.</td>
<td>The peaks due to Montelukast Would be completely resolved from any other peaks. Excipient blend, blank and known Impurities Would not show any peak at the RT of the Montelukast.</td>
</tr>
<tr>
<td>Specificity - Forced degradation study</td>
<td>----</td>
<td>The forced degradation studies would be performed using 1 N HCl, 1N NaOH, 30% H2O2. Also effect of humidity, temperature and photolysis would be checked. The studies shall be performed on excipient blend, API and Tablet powder separately.</td>
<td>Degrading peaks Would be completely separated from the Montelukast peak. 10% – 30% degradation in at least one stress condition shall be achieved. Peak purity angle of peak due to Montelukast would not be more than the peak purity threshold in untreated and treated samples which will states that peak purity passes.</td>
</tr>
<tr>
<td>Linearity and Range</td>
<td>----</td>
<td>50 % to 150%</td>
<td>Correlation coefficient between concentration and area of peak and its range from 50% - 150% of sample concentration would not be less than 0.99.</td>
</tr>
<tr>
<td>System Precision</td>
<td>----</td>
<td>----</td>
<td>% RSD peak areas for five injection of standard would NMT 2.0</td>
</tr>
<tr>
<td>Method Precision</td>
<td>----</td>
<td>----</td>
<td>In this test % assay value calculate and percentage RSD would not exceed 2.0 % value.</td>
</tr>
</tbody>
</table>
Table 7………. continued

<table>
<thead>
<tr>
<th>Validation parameter</th>
<th>Specification</th>
<th>Range of study</th>
<th>Acceptance criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intermediate Precision (IP)</td>
<td>----</td>
<td>different system, different column of same make but different serial no.</td>
<td>% RSD of the assay results of six test Samples would not be more than 2.0. % RSD of the assay results of twelve test Samples (six of Precision of Method and six of Intermediate-Precision (IP)) would not be more than 2.0.</td>
</tr>
<tr>
<td>Recovery ie.accuracy (%Recovery)</td>
<td>----</td>
<td>At 50%, 100% &amp; 150 % (for it Sample concentration to be consider)</td>
<td>Result found for this test would be in range of 98.00 % -102.00 %. (it shows accuracy of method) For all levels percentage RSD would not exceed than 2.00 %</td>
</tr>
<tr>
<td>Filter Validation</td>
<td>----</td>
<td>Unfiltered and filtered Sample.</td>
<td>The difference between test Sample filtered and test Sample unfiltered would not be more than 2.0%.</td>
</tr>
<tr>
<td>Robustness – change in column lot</td>
<td>15 centi meter X 4.6 mili-meter 5 µ, C-18, (Waters Symmetry)</td>
<td>Same make, different serial no.</td>
<td>% RSD between results obtained with changed condition and six results of method precision would not be more than 2.0.</td>
</tr>
<tr>
<td>Robustness compartment temp. changes for column (± 5°C)</td>
<td>30°C</td>
<td>Lower temp- 25 °C &amp; High temp.-35 ºC</td>
<td>% RSD between results obtained with changed condition and six results of method precision would not be more than 2.0.</td>
</tr>
<tr>
<td>Robustness change in pH - buffer(± 0.2) Unit</td>
<td>6.00</td>
<td>5.80 and 6.20</td>
<td>%RSD between results obtained with changed condition and six results of method precision would not be more than 2.0.</td>
</tr>
</tbody>
</table>
### Table 7………. continued

<table>
<thead>
<tr>
<th>Validation parameter</th>
<th>Specification</th>
<th>Range of study</th>
<th>Acceptance criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Robustness – change in wavelength (± 2 Nanometer)</td>
<td>225 Nanometer</td>
<td>223 Nanometer and 227 Nanometer</td>
<td>% RSD between results obtained with changed condition and six results of method precision would not be more than 2.0.</td>
</tr>
<tr>
<td>Stability of analytical Sample</td>
<td>At Room Temperature</td>
<td>Evaluate the Sample stability for seven days for test Sample and standard Sample (Reference Sample)</td>
<td>The Sample is considered stable, till the time point where the % RSD of the stored and initial prepared test and standard Sample (Reference Sample) is NMT 2.0.</td>
</tr>
</tbody>
</table>
5.1.2 EXPERIMENTAL PLAN AND DATA EVALUATION FOR
LOPINAVIR & RITONAVIR
SOFT GELATIN CAPSULES.

1. Specificity:
Experiment: selectivity

Buffer Preparation:
Dissolved 34.135 g Dihydrogen Potassium phosphate in to 5 liter water. Make pH for this buffer Sample to 3.04 ± 0.05 Unit with help of OPA (O-phosphoric Acid) and filter this from Zero point Forty five (0.4 micro meter) um nylon filters.

Making of Dilute Orthophosphoric acid Sample:
Take 200 milliliter of volumetric flask added 20 milliliter of OPA (O-phosphoric Acid) and diluted this flask to its ring mark and homogeneously mixed

Making of Solvent Mixture Sample:
Prepared mixture of ACN (Acetonitrile) and Methanol in composition of 4000:1000 ml

Mobile Phase:
Mixed a The Mixture of filtered buffer (Degassed) and Solvent Mixture in proportion of 2250:2750.

Diluent Sample:
I. Methanol
II. Mobile Phase.

Chromatographic Conditions:
System Controlling Parameter
Name of system : HPLC Photo Diode array detector)
Used Column : SB C-18, 50 millimetre x 4.6 millimetre, 3.5 µm. (Zorbax)
System flow- rate : 1.0 milliliter / minute
Absorption Wavelength : 210 Nano-meter
Temperature of Column oven : 25°C
Volume at time of injection : 10 µl
Run time of injection : 15 Minutes
Mode type For Analysis : Isocratic

Making of Lopinavir Stock Sample:
Weighed and transferred 35.01 mg of Lopinavir standard in 50 milli liter of flask to it added 35 milliliter of Methanol dissolved and make up it to volume (ring mark) of flask with Methyl alcohol (Methanol).

Making of Ritonavir Stock Sample:
Weighed and transferred 35.10 mg of Ritonavir standard in 200 milli liter of flask to it added 150 milliliter of Methanol dissolved and make up It to volume (ring mark) of flask with Methyl alcohol (Methanol).

Making of Standard Sample (Reference Sample) :
Added 5.0 milliliter each of stock Sample to 50.0 milliliter flask (Volumetric) and diluted to 50 milliliter it to volume (ring mark) of flask with help of diluent i.e. Mobile-phase.

Making of Diluent blank Sample:
In 50.0 milliliter of flask added 5.0 milliliter of methyl alcohol to it mixed mobile phase to ring mark of flask which used as blank.

Making of Placebo Sample:
Transferred 0.6508 g of placebo in 250 milli liter of flask. Added 170 ml of Methanol Sonicated it through occasional Slight Shaking for 25 Minutes diluted final volume of flask with help of Methyl alcohol (Methanol-Solvent) and mixed properly. By filtration of Sample from Zero point Forty five (0.4 micro meter) Micron size filter discarded first 3 milliliter filtrate and Again diluted to 50 milliliter volumetric flask with mobile phase having 5 milliliter of this Sample (stock Sample).
Making of Placebo with shell:
Transferred one placebo capsule in 200 milli liter of flask. Then by adding about 35 milliliter diluent-mobile phase give wave sonication of 15 minute to dispersed Shell, then add 100 ml Methanol Sonicate it through occasional Slight Shaking for 35 min, diluted its final volume of flask with help of Methyl alcohol (Methanol-Solvent) and mixed properly & By filtration of Sample from Zero point Forty five (0.4 micro meter) Micron size filter(Used syringe filter) discarded first 3.0 milliliter solution and Again diluted to 50 milliliter volumetric flask with mobile phase having 5 milliliter of this Sample (stock Sample).

Making Sample: (Stock for Lopinavir)
Weighed 35.10 milligram of standard (Lopinavir) in 50 milli liter of flask to it add 35 milliliter of Methanol dissolve and make up it to volume (ring mark) of flask with Methyl alcohol (Methanol).

Making of Ritonavir Stock Sample:
Weighed and transferred 35.00 mg of Ritonavir standard in 200 milli liter of flask to it add 170 milli liter of Methanol dissolve and make up it to volume (ring mark) of flask with Methyl alcohol (Methanol).

Making of Standard Sample (Reference Sample):
Added 5 ml each of stock Sample to 50.0 milliliter flask (Volumetric) and diluted to 50 milliliter it to volume (ring mark) of flask using diluent i.e. Mobile-phase

Making of Lopinavir Related Compound -A Sample:
Weighed and transferred 6.01 mg of Lopinavir Related Compound -A in 100 milli liter of flask to it add 50 milli liter of Methanol dissolve and make up it to volume (ring mark) of flask with Methyl alcohol (Methanol). Again diluted 5 milliliter of respective solution to 200 ml with mobile phase.

Making of Lopinavir Related Compound -B Sample:
Weighed and transferred 6.05 mg of Lopinavir Related Compound -B in 100 milli liter of flask to it add 50 milli liter of Methanol dissolve and make up it to volume
(ring mark) of flask with Methyl alcohol (Methanol). Again diluted 5 milliliter of respective solution to 200 ml with mobile phase.

**Making of Lopinavir Related Compound -C Sample:**
Weighed and transferred 6.08 mg of Lopinavir Related Compound -C in 100 milliliter of flask to it add 50 milli liter of Methanol dissolve and make up it to volume (ring mark) of flask with Methyl alcohol (Methanol). Again diluted 5 milliliter of respective solution to 200 ml with mobile phase.

**Making of Lopinavir Related Compound -D Sample:**
Weighed and transferred 6.02 mg of Lopinavir Related Compound -D in 100 milliliter of flask to it add 50 milli liter of Methanol dissolve and make up it to volume (ring mark) of flask with Methyl alcohol (Methanol). Again diluted 5 milliliter of respective solution to 200 ml with mobile phase.

**Making of Sample:**
Weighed and transferred 69.15 mg of Ritonavir standard, 280.12 mg of Lopinavir Standard in 100 milliliter of flask to it add 70 milli liter of Methanol dissolve and make up it to volume (ring mark) of flask with Methyl alcohol (Methanol). Again diluted 5.0 milliliter of this Sample, 10.0 milli liter each related compound to 200 milliliter by mobile phase.

The sequence of samples for selectivity given as

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>No. of Injections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluent Blank Sample</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Standard Sample (Reference Sample)</td>
<td>Replicate 5</td>
</tr>
<tr>
<td>Diluent Blank Sample</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Placebo Sample</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Capsule Shell Sample</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Sample solution- Rel.comp : A</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Sample solution- Rel.comp : B</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Sample solution- Rel.comp : C</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Sample solution- Rel.comp : D</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Check standard</td>
<td>Replicate 1</td>
</tr>
</tbody>
</table>
**Data evaluation:**

Record each chromatogram. All the injections will be processed at the wavelength provided in the method and selectivity will be demonstrated with regards to non-interference from peaks due to Diluent blank Sample, excipient blend Sample and known impurities with Lopinavir & Ritonavir peak. The peak purity will be determined for Lopinavir & Ritonavir peak.
2. Forced degradation:

Experiment:
Buffer Preparation:
Buffer Preparation: (solution A)
Dissolved 34.135 g Dihydrogen Potassium phosphate in to 5 liter water. Make pH for this buffer Sample to 3.02 ± 0.05 Unit with help of OPA (O-phosphoric Acid) and filter this from Zero point Forty five (0.4 micro meter) um nylon filters.

Making of Dilute Orthophosphoric acid Sample:
Take 200 milliliter of volumetric flask added 20 milliliter of OPA (O-phosphoric Acid) and diluted this flask to its ring mark and homogeneously mixed

Making of Solvent Mixture Sample:
Prepared mixture of ACN (Acetonitrile) and Methanol in composition of 4000:1000 milliliter
Mobile Phase:
Mixed a The Mixture of filtered solution A (Degassed) and Solvent Mixture in proportion of 2250:2750.

Diluent Sample:
I. Methanol
II. Mobile Phase.

Degrading agents will be added separately to Placebo Sample and Lopinavir & Ritonavir Soft gelatin capsules. Degrading agents will be 1N HCl (Acid degradation), 1N NaOH (Base degradation), 30% Hydrogen peroxide (Oxidative degradation), Heat Sample (60°C), Photolysis.
System Controlling Parameter

Name of system : HPLC Photo Diode array detector
Used Column : SB C-18, 50 millimetre x 4.6 millimetre, 3.5 μm. (Zorbax)
System flow-rate : 1.0 milliliter / minute
Absorption Wavelength : 210 Nano-meter
Temperature of Column oven : 25°C
Volume at time of injection : 10 μl
Run time of injection : 15 Minutes
Mode type For Analysis : Isocratic

Making of Lopinavir Stock Sample:
Transferred 35.18 mg of Lopinavir standard in 50 milliliter of flask to it added 35 milli liter of Methanol dissolved and make up it to volume (ring mark) of flask by Methyl alcohol (Methanol).

Making of Ritonavir Stock Sample:
Transferred 35.10 mg of Ritonavir standard in 200 milli liter of flask to it added 150 milli liter of Methanol dissolved and make up It to volume (ring mark) of flask by Methyl alcohol (Methanol).

Making of Standard Sample (Reference Sample) :
Added 5 ml each of stock Sample to 50.0 milli liter flask (Volumetric) and diluted to 50 milli liter it to volume (ring mark) of flask using diluent i,e Mobile-phase

Making of Samples for forced degradation studies (Placebo Sample)

1. Placebo Sample - Untreated:
Transferred 0.6608 g of placebo in 250 milliliter of flask. Added 170 ml of Methanol Sonicated it through occasional Slight Shaking for about 30 Minutes diluted to final volume of flask by Methyl alcohol (Methanol solvent) & homogeneously mixed. By filtration of Sample from Zero point Forty five (0.4 micro meter) Micron size filter discarded first 3.0 milliliter solution and Again
diluted to 50 milliliter volumetric flask with mobile phase having 5 milliliter of this Sample (stock Sample).

2. Placebo Sample – Acid Degradation:
Transferred 0.6548 g of placebo in 250 milliliter of flask. Added 170 ml of Methanol, Sonicated it through occasional Slight Shaking for about 30 min, to it added 2.0 milliliter 1N HCl, Kept the flask as it 1 hours at room temperature. Neutralized the Sample with 1N NaOH and diluted final volume of flask with help of Methyl alcohol (Methanol solvent) & homogeneously mixed. By filtration of Sample from Zero point Forty five (0.4 micro meter) Micron nylon size filter discarded first 3.0 milliliter solution and Again diluted to 50 milliliter volumetric flask with mobile phase having 5 milliliter of this Sample (stock Sample). A diluent blank Sample (without Placebo) would be prepared in similar way.

3. Placebo Sample – Base Degradation:
Transferred 0.6567 g of placebo in 250 milliliter of flask. Added 170 ml of Methanol, Sonicated it through occasional Slight Shaking for 25 Minutes to it added 2.0 milliliter 1N NaOH, Kept the flask as it 1 hours at room temperature. Neutralized the Sample with 1N Hydro-chloric acid and diluted to final volume of flask with help of Methyl alcohol (Methanol solvent) & homogeneously mixed. By filtration of Sample from Zero point Forty five (0.4 micro meter) Micron nylon size filter discarded first 3.0 milliliter solution and Again diluted to 50 milliliter volumetric flask with mobile phase having 5 milliliter of this Sample (stock Sample). A diluent blank Sample (without Placebo) would be prepared in similar way.

4. Placebo Sample – Peroxide Degradation:
Transferred 0.6678 g of placebo in 250 milliliter of flask. Added 170 ml of Methanol, Sonicated it through occasional Slight Shaking for 25 Minutes to it added 2.0 milliliter hydrogen peroxide (30.0 percent) , Kept the flask as it 1 hours at nominal room temperature. Diluted to final volume of flask with help of Methyl alcohol (Methanol solvent) & homogeneously mixed. By filtration of Sample from Zero point Forty five (0.4 micro meter) Micron size filter discarded first 3.0
milliliter solution and Again diluted to 50 milliliter volumetric flask with mobile phase having 5 milliliter of this Sample (stock Sample).

A diluent blank Sample (without Placebo) would be prepared in similar way.

5. Placebo Sample – Heat Sample State Degradation:
Transferred 0.6658 g of placebo in 250 milliliter of flask. Added 170 ml of Methanol, Sonicated it through occasional Slight Shaking for 25 Minutes to it and heat this Sample at 60°C for 30 minute equilibrate to ambient temperature (Room temperature) diluted to final volume of flask with Methyl alcohol (Methanol solvent) & homogeneously mixed. By filtration of Sample from Zero point Forty five (0.4 micro meter) Micron nylon size Filters discarded first 3.0 milliliter solution and Again diluted to 50 milliliter volumetric flask with mobile phase having 5 milliliter of this Sample (stock Sample).

6. Placebo Sample – Photolytic Degradation:
Transferred 0.6545 g of treated placebo in 250 milliliter of flask. Added 170 ml of Methanol Sonicated it through occasional Slight Shaking for 25 Minutes to it diluted to final volume of flask with help of Methyl alcohol (Methanol solvent) & homogeneously mixed. By filtration of Sample from Zero point Forty five (0.4 micro meter) Micron size filters discarded first 3.0 milliliter solution and Again diluted to 50 milliliter volumetric flask with mobile phase having 5 milliliter of this Sample (stock Sample).
Making of Test Samples for forced degradation studies (Samples)

1. Sample - Untreated:
Transferred 0.9895 g of Capsules Content in 250 milliliter of flask. Added 170 ml of Methanol Sonicated it through occasional Slight Shaking for 25 Minutes diluted to final volume of flask with Methyl alcohol (Methanol solvent) & homogeneously mixed. By filtration of Sample from Zero point Forty five (0.4 micro meter) Micron nylon size filters discarded first 3.0 milliliter solution and Again diluted to 50 milliliter volumetric flask with mobile phase having 5 milliliter of this Sample (stock Sample).

2. Sample – Acid Degradation:
Transferred 0.9978 g of Capsules Content in 250 milliliter of flask. Added 170 ml of Methanol, Sonicated it through occasional Slight Shaking for about 30 Minutes to it added 2.0 milliliter 1N HCl, Kept the flask for 1 hours at room temperature. Neutralized the Sample with 1N NaOH and diluted to final volume of flask with help of Methyl alcohol (Methanol solvent) & homogeneously mixed. By filtration of Sample from Zero point Forty five (0.4 micro meter) Micron size filters discarded first 3.0 milliliter solution and Again diluted to 50 milliliter volumetric flask with mobile phase having 5 milliliter of this Sample (stock Sample). Blank Sample (without Placebo) would be prepared in similar way.

3. Sample – Base Degradation:
Transferred 0.9999 g of Capsules Content in 250 milliliter of flask. Added 170 ml of Methanol, Sonicated it through occasional Slight Shaking for 25 Minutes to it added 2.0 milliliter 1N NaOH, Kept the flask as it 1 hours at room temperature. Neutralized the Sample with 1N Hydro-chloric acid and diluted to final volume of flask with help of Methyl alcohol (Methanol solvent) & homogeneously mixed. By filtration of Sample from Zero point Forty five (0.4 micro meter) Micron nylon size Filters discarded first 3.0 milliliter solution and Again diluted to 50 milliliter volumetric flask with mobile phase having 5 milliliter of this Sample (stock Sample).
Blank Sample (without Placebo) would be prepared in similar way.

4. Sample – Peroxide Degradation:
Transferred 1.0128 g of Capsules Content in 250 milli liter of flask. Added 170 ml of Methanol, Sonicated it through occasional Slight Shaking for 25 Minutes to it added 2.0 milliliter 30 % Hydrogen peroxide to the flask. Kept the flask for 1 hours at room temperature. Diluted to final volume of flask with Methyl alcohol (Methanol solvent) & homogeneously mixed. By filtration of Sample from Zero point Forty five (0.4 micro meter) Micron nylon size Filters discarded first 3.0 milliliter solution and again diluted to 50 milliliter volumetric flask with mobile phase having 5 milliliter of this Sample (stock Sample).

Blank Sample (without Placebo) would be prepared in similar way.

5. Sample – Heat Degradation:
Transferred 0.9997 g of Capsules Content in 250 milli liter of flask. Added 170 ml of Methanol, Sonicated it through occasional Slight Shaking for 25 Minutes to it and heat this Sample at 60°C for 30 minute equilibrate to ambient temperature (Room temperature) diluted to final volume of flask with help of Methyl alcohol (Methanol solvent) & homogeneously mixed. By filtration of Sample from Zero point Forty five (0.4 micro meter) Micron size Filters discarded first 3.0 milliliter solution and again diluted to 50 milliliter volumetric flask with mobile phase having 5 milliliter of this Sample (stock Sample).

6. Sample – Photolytic Degradation:
Transferred 1.0015 g of Capsules Content in 250 milliliter of flask. Added 170 ml of Methanol Sonicated it through occasional Slight Shaking for 25 Minutes to it diluted to final volume of flask with help of Methyl alcohol (Methanol solvent) & homogeneously mixed. By filtration of Sample from Zero point Forty five (0.4 micro meter) Micron nylon size Filters discarded first 3.0 milliliter solution and again diluted to 50 milliliter volumetric flask with mobile phase having 5 milliliter of this Sample (stock Sample).
The sequence of samples for Forced Degradation given as:

An example of sequence for Forced Degradation

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>No. of Injections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank Sample</td>
<td>1</td>
</tr>
<tr>
<td>Standard Sample (Reference Sample)</td>
<td>5</td>
</tr>
<tr>
<td>Blank Sample (Acid degradation)</td>
<td>1</td>
</tr>
<tr>
<td>Blank Sample (Base degradation)</td>
<td>1</td>
</tr>
<tr>
<td>Blank Sample (Peroxide degradation)</td>
<td>1</td>
</tr>
<tr>
<td>Placebo Sample-Untreated</td>
<td>1</td>
</tr>
<tr>
<td>Placebo Sample (Acid degradation)</td>
<td>1</td>
</tr>
<tr>
<td>Placebo Sample (Base degradation)</td>
<td>1</td>
</tr>
<tr>
<td>Placebo Sample (Peroxide degradation)</td>
<td>1</td>
</tr>
<tr>
<td>Placebo Sample (Sample State)</td>
<td>1</td>
</tr>
<tr>
<td>Placebo Sample (photolytic)</td>
<td>1</td>
</tr>
<tr>
<td>Sample (Acid degradation)</td>
<td>1</td>
</tr>
<tr>
<td>Bracketing standard-1</td>
<td>1</td>
</tr>
<tr>
<td>Sample (Base degradation)</td>
<td>1</td>
</tr>
<tr>
<td>Sample (Peroxide degradation)</td>
<td>1</td>
</tr>
<tr>
<td>Sample (Sample State)</td>
<td>1</td>
</tr>
<tr>
<td>Sample (photolytic)</td>
<td>1</td>
</tr>
<tr>
<td>Bracketing standard-2</td>
<td>1</td>
</tr>
</tbody>
</table>

Data evaluation:

Record each chromatogram. All the injections will be processed at the wavelength provided in the method. Peak purity demonstrated for peak of Lopinavir & Ritonavir. Calculated the % degradation for each state and reported.
3. Linearity:

Experiment:

Buffer Preparation:

Buffer Preparation: (solution A)
Dissolved 34.135 g Dihydrogen Potassium phosphate in to 5 liter water. Make pH for this buffer Sample to 3.02 ± 0.05 Unit with help of OPA (O-phosphoric Acid) and filter this from Zero point Forty five (0.4 micro meter) um nylon filters.

Making of Dilute Orthophosphoric acid Sample:
Take 200 milliliter of volumetric flask added 20 milliliter of OPA (O-phosphoric Acid) and diluted this flask to its ring mark and homogeneously mixed

Making of Solvent Mixture Sample:
Prepared mixture of ACN (Acetonitrile) and Methanol in composition of 4000:1000 milliliter

Mobile Phase:
Mixed a The Mixture of filtered solution A (Degassed) and Solvent Mixture in proportion of 2250:2750.

Diluent Sample:
   I. Methanol
   II. Mobile Phase.

System Controlling Parameter
Name of system : HPLC
Used Column : SB C-18, 50 millimetre x 4.6 millimetre, 3.5 µm. (Zorbax)
System flow-rate : 1.0 milliliter / minute
Absorption Wavelength : 210 Nano-meter
Temperature of Column oven : 25°C
Volume at time of injection : 10 µl
Run time of injection : 15 Minutes
Mode type For Analysis : Isocratic

Making of Lopinavir Stock Sample:
Transferred 35.25 mg of Lopinavir standard in 50 milliliter of flask to it added 35 milli liter of Methanol dissolved and make up It to volume (ring mark) of flask by Methyl alcohol (Methanol).

**Making of Ritonavir Stock Sample:**
Transferred 35.74 mg of Ritonavir standard in 200 milli liter of flask to it added 150 milli liter of Methanol dissolved and make up It to volume (ring mark) of flask by Methyl alcohol (Methanol).

**Making of Standard Sample (Reference Sample) :**
Added 5 ml each of stock Sample to 50.0 milliliter flask (Volumetric) and diluted to 50 milliliter it to volume (ring mark) of flask using diluent i,e Mobile-phase

**Making of Linearity Stock Sample:**
Weighed and transferred 140.21 milligram standard (Lopinavir) and 34.52 milligram standard (Ritonavir) to 100 milliliter of flask to it added 70 milli liter of Methanol dissolved and make up It to volume (ring mark) of flask with Methyl alcohol (Methanol).

**Making of Linearity Levels Sample:**

<table>
<thead>
<tr>
<th>Linearity Level</th>
<th>Volume of stock Sample Added in milliliter</th>
<th>Dilution in milliliter</th>
<th>Concentration of Ritonavir (ppm)</th>
<th>Concentration of Lopinavir(ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level – 1</td>
<td>4.0</td>
<td>100</td>
<td>7.0</td>
<td>28.0</td>
</tr>
<tr>
<td>Level – 2</td>
<td>6.0</td>
<td>100</td>
<td>10.5</td>
<td>42.0</td>
</tr>
<tr>
<td>Level – 3</td>
<td>8.0</td>
<td>10</td>
<td>14.0</td>
<td>56.0</td>
</tr>
<tr>
<td>Level – 4</td>
<td>10.0</td>
<td>100</td>
<td>17.5</td>
<td>70.0</td>
</tr>
<tr>
<td>Level – 5</td>
<td>11.0</td>
<td>100</td>
<td>19.25</td>
<td>77.0</td>
</tr>
<tr>
<td>Level – 6</td>
<td>13.0</td>
<td>100</td>
<td>22.75</td>
<td>91.0</td>
</tr>
<tr>
<td>Level – 7</td>
<td>15.0</td>
<td>100</td>
<td>26.25</td>
<td>105.0</td>
</tr>
</tbody>
</table>

Table 8 : Linearity Levels
### An example of Sequence for Linearity

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>No. of Injections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluent Blank Sample</td>
<td>2</td>
</tr>
<tr>
<td>Standard Sample (Reference Sample)</td>
<td>5</td>
</tr>
<tr>
<td>Linearity Level 1</td>
<td>3</td>
</tr>
<tr>
<td>Linearity Level 2</td>
<td>3</td>
</tr>
<tr>
<td>Linearity Level 3</td>
<td>3</td>
</tr>
<tr>
<td>Bracketing standard_1</td>
<td>2</td>
</tr>
<tr>
<td>Linearity Level 4</td>
<td>3</td>
</tr>
<tr>
<td>Linearity Level 5</td>
<td>3</td>
</tr>
<tr>
<td>Bracketing standard_2</td>
<td>2</td>
</tr>
</tbody>
</table>

**Data evaluation:**

Linearity graph Plotted of average area at each level against the concentration (ppm) and determined the correlation coefficient.
4. Precision:

Precision of Method and System Precision:

Experiment:

**Buffer Preparation: (solution A)**

Dissolved 34.135 g Dihydrogen Potassium phosphate in to 5 liter water. Make pH for this buffer Sample to 3.02 ± 0.05 Unit with help of OPA (O-phosphoric Acid) and filter this from Zero point Forty five (0.4 micro meter) um nylon filters.

**Making of Dilute Orthophosphoric acid Sample:**

Take 200 milliliter of volumetric flask added 20 milliliter of OPA (O-phosphoric Acid) and diluted this flask to its ring mark and homogeneously mixed

**Making of Solvent Mixture Sample:**

Prepared mixture of ACN (Acetonitrile) and Methanol in composition of 4000:1000 milliliter

**Mobile Phase:**

Mixed a The Mixture of filtered solution A (Degassed) and Solvent Mixture in proportion of 2250:2750.

**Diluent Sample:**

I. Methanol
II. Mobile Phase.

**System Controlling Parameter**

- Name of system : HPLC
- Used Column : SB C-18, 50 millimetre x 4.6 millimetre, 3.5 µm. (Zorbax)
- System flow-rate : 1.0 milliliter / minute
- Absorption Wavelength : 210 Nano-meter
- Temperature of Column oven : 25°C
- Volume at time of injection : 10 µl
- Run time of injection : 15 Minutes
- Mode type For Analysis : Isocratic
Making of Lopinavir Stock Sample:
Transferred 35.25 mg of Lopinavir standard in 50 milliliter of flask to it added 35 milli liter of Methanol dissolved and make up It to volume (ring mark) of flask by Methyl alcohol (Methanol).

Making of Ritonavir Stock Sample:
Transferred 35.74 mg of Ritonavir standard in 200 milli liter of flask to it added 150 milli liter of Methanol dissolved and make up It to volume (ring mark) of flask by Methyl alcohol (Methanol).

Making of Standard Sample (Reference Sample) :
Added 5 ml each of stock Sample to 50.0 milliliter flask (Volumetric) and diluted to 50 milliliter it to volume (ring mark) of flask using diluent i,e Mobile-phase

Making of sample - 1:
Transferred 0.9989 g of Capsules Content in 250 milliliter of flask. Added 170 ml of Methanol Sonicated it through occasional Slight Shaking for 30 Minutes diluted to final volume of flask with help of Methyl alcohol, mixed properly, By filtration of Sample from Zero point Forty five (0.4 micro meter) micron size Filters discarded first 3.0 milliliter solution and again diluted to 50 milliliter volumetric flask with mobile phase having 5 milliliter of this Sample (stock Sample).

Making of Sample – 2:
Transferred 0.9990 g of Capsules Content in 250 milliliter of flask. Added 170 ml of Methanol Sonicated it through occasional Slight Shaking for 30 Minutes diluted to final volume of flask with help of Methyl alcohol, mixed properly, By filtration of Sample from Zero point Forty five (0.4 micro meter) micron size Filters discarded first 3.0 milliliter solution and again diluted to 50 milliliter volumetric flask with mobile phase having 5 milliliter of this Sample (stock Sample).

Making of Sample - 3:
Transferred 0.9989 g of Capsules Content in 250 milliliter of flask. Added 170 ml of Methanol Sonicated it through occasional Slight Shaking for 30 Minutes diluted to
final volume of flask with help of Methyl alcohol, mixed properly, By filtration of Sample from Zero point Forty five (0.4 micro meter) micron size Filters discarded first 3.0 milliliter solution and again diluted to 50 milliliter volumetric flask with mobile phase having 5 milliliter of this Sample (stock Sample).

**Making of Sample - 4:**
Transferred 1.0054 g of Capsules Content in 250 milliliter of flask. Added 170 ml of Methanol Sonicated it through occasional Slight Shaking for 30 Minutes diluted to final volume of flask with help of Methyl alcohol, mixed properly, By filtration of Sample from Zero point Forty five (0.4 micro meter) micron size Filters discarded first 3.0 milliliter solution and again diluted to 50 milliliter volumetric flask with mobile phase having 5 milliliter of this Sample (stock Sample).

**Making of Sample - 5:**
Transferred 0.9896 g of Capsules Content in 250 milliliter of flask. Added 170 ml of Methanol Sonicated it through occasional Slight Shaking for 30 Minutes diluted to final volume of flask with help of Methyl alcohol, mixed properly, By filtration of Sample from Zero point Forty five (0.4 micro meter) micron size Filters discarded first 3.0 milliliter solution and again diluted to 50 milliliter volumetric flask with mobile phase having 5 milliliter of this Sample (stock Sample).

**Making of Sample - 6:**
Transferred 0.9994 g of Capsules Content in 250 milliliter of flask. Added 170 ml of Methanol Sonicated it through occasional Slight Shaking for 30 Minutes diluted to final volume of flask with help of Methyl alcohol, mixed properly, By filtration of Sample from Zero point Forty five (0.4 micro meter) micron size Filters discarded first 3.0 milliliter solution and again diluted to 50 milliliter volumetric flask with mobile phase having 5 milliliter of this Sample (stock Sample).
An example of Sequence for Method Precision

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>No. of Injections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluent Blank Sample</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Standard Sample (Reference Sample)</td>
<td>Replicates 5</td>
</tr>
<tr>
<td>Test Sample – 1</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Test Sample – 2</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Test Sample – 3</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Test Sample – 4</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Test Sample – 5</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Test Sample – 6</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Bracketing standard</td>
<td>Replicate 1</td>
</tr>
</tbody>
</table>

Data evaluation:
The % assay will be calculated and reported along with the SD and % RSD of the six test Samples.
5. Recovery or accuracy (% Recovery):

**Experiment:**

**Buffer Preparation: (solution A)**

Dissolved 34.055 g Dihydrogen Potassium phosphate in to 5 liter water. Make pH for this buffer Sample to 3.02 ± 0.05 Unit with help of OPA (O-phosphoric Acid) and filter this from Zero point Forty five (0.4 micro meter) um nylon filters.

**Making of Dilute Orthophosphoric acid Sample:**

Take 200 milliliter of volumetric flask added 20 milliliter of OPA (O-phosphoric Acid) and diluted this flask to its ring mark and homogeneously mixed

**Making of Solvent Mixture Sample:**

Prepared mixture of ACN (Acetonitrile) and Methanol in composition of 4000:1000 milliliter

**Mobile Phase:**

Mixed a The Mixture of filtered solution A (Degassed) and Solvent Mixture in proportion of 2250:2750.

**Diluent Sample:**

- I. Methanol
- II. Mobile Phase.

**System Controlling Parameter**

- Name of system : HPLC
- Used Column : SB C-18, 50 millimetre x 4.6 millimetre, 3.5 µm. (Zorbax)
- System flow- rate : 1.0 milliliter / minute
- Absorption Wavelength : 210 Nano-meter
- Temperature of Column oven : 25°C
- Volume at time of injection : 10 µl
- Run time of injection : 15 Minutes
- Mode type For Analysis : Isocratic
Making of Lopinavir Stock Sample:
Transferred 35.45 mg of Lopinavir standard in 50 milliliter of flask to it added 35 milli liter of Methanol dissolved and make up it to volume (ring mark) of flask by Methyl alcohol (Methanol).

Making of Ritonavir Stock Sample:
Transferred 35.05 mg of Ritonavir standard in 200 milli liter of flask to it added 150 milli liter of Methanol dissolved and make up it to volume (ring mark) of flask by Methyl alcohol (Methanol).

Making of Standard Sample (Reference Sample) :
Added 5 ml each of stock Sample to 50.0 milli liter flask (Volumetric) and diluted to 50 milli liter it to volume (ring mark) of flask using diluent i,e Mobile-phase.

Making of Recovery ie.accuracy Levels:
Weighed and transferred accurately, in triplicate at each level, about 0.6330 g of placebo and added to it Lopinavir & Ritonavir drug at each level of the working concentration.

The Making of Recovery ie.accuracy Samples is given in table 9.
### Table 9: Dilutions for Recovery ie.accuracy for Lopinavir & Ritonavir.

<table>
<thead>
<tr>
<th>Level</th>
<th>Lopinavir Drug Added in mg</th>
<th>Ritonavir Drug Added in mg</th>
<th>Placebo added in g</th>
<th>Approximately Concentration of Lopinavir in ppm</th>
<th>Approximately Concentration of Ritonavir in ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>First Level</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>70.66</td>
<td>28.46</td>
<td>0.6345</td>
<td>28</td>
<td>11.4</td>
</tr>
<tr>
<td></td>
<td>69.90</td>
<td>28.15</td>
<td>0.6356</td>
<td>28</td>
<td>11.4</td>
</tr>
<tr>
<td></td>
<td>70.19</td>
<td>28.24</td>
<td>0.6357</td>
<td>28</td>
<td>11.4</td>
</tr>
<tr>
<td><strong>Second Level</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>175.35</td>
<td>43.25</td>
<td>0.6326</td>
<td>70</td>
<td>17.5</td>
</tr>
<tr>
<td></td>
<td>175.15</td>
<td>43.56</td>
<td>0.6338</td>
<td>70</td>
<td>17.5</td>
</tr>
<tr>
<td></td>
<td>175.22</td>
<td>43.35</td>
<td>0.6345</td>
<td>70</td>
<td>17.5</td>
</tr>
<tr>
<td><strong>Third Level</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>262.31</td>
<td>67.53</td>
<td>0.6352</td>
<td>105</td>
<td>27.0</td>
</tr>
<tr>
<td></td>
<td>262.54</td>
<td>67.52</td>
<td>0.6314</td>
<td>105</td>
<td>27.0</td>
</tr>
<tr>
<td></td>
<td>262.31</td>
<td>67.56</td>
<td>0.6344</td>
<td>105</td>
<td>27.0</td>
</tr>
</tbody>
</table>

**Making of Recovery ie.accuracy Levels 50% - 1:**
Weighed and transferred 0.6345 g of placebo, 70.66 mg of Lopinavir drug, 28.46 mg Ritonavir drug in 250 milliliter of flask to it added 150 milliliter of Methanol. Sonicated for 30 Minutes, diluted to final volume of flask by Methyl alcohol and mixed properly. By filtration of Sample from Zero point Forty five (0.4 micro meter) Micron Filters discarded first 3.0 milliliter solution and Again diluted to 50 milliliter volumetric flask with mobile phase having 5 milliliter of this Sample (stock Sample).

**Making of Recovery ie.accuracy Levels 50% - 2:**
Weighed and transferred 0.6356 g of placebo, 69.90 mg of Lopinavir drug, 28.15 mg Ritonavir drug in 250 milliliter of flask to it added 150 milliliter of Methanol. Sonicated for 30 Minutes, diluted to final volume of flask by Methyl alcohol and mixed properly. By filtration of Sample from Zero point Forty five (0.4 micro meter) Micron Filters discarded first 3.0 milliliter solution and Again diluted to 50 milliliter volumetric flask with mobile phase having 5 milliliter of this Sample (stock Sample).

**Making of Recovery ie.accuracy Levels 50% - 3:**
Weighed and transferred 0.6357 g of placebo, 70.19 mg of Lopinavir drug, 28.24 mg of Ritonavir drug in 250 milliliter of flask to it added 150 milliliter of Methanol. Sonicated for 30 Minutes, diluted to final volume of flask by Methyl alcohol and mixed properly, By filtration of Sample from Zero point Forty five (0.4 micro meter) Micron Filters discarded first 3.0 milliliter solution and Again diluted to 50 milliliter volumetric flask with mobile phase having 5 milliliter of this Sample (stock Sample).

**Making of Recovery ie.accuracy Levels 100% - 1:**
Weighed and transferred 0.6326 g of placebo, 175.35 mg of Lopinavir drug, 43.25 mg of Ritonavir drug in 250 milliliter of flask to it added 150 milliliter of Methanol. Sonicated for 30 Minutes, diluted to final volume of flask by Methyl alcohol and mixed properly, By filtration of Sample from Zero point Forty five (0.4 micro meter) Micron Filters discarded first 3.0 milliliter solution and Again diluted to 50 milliliter volumetric flask with mobile phase having 5 milliliter of this Sample (stock Sample).

**Making of Recovery ie.accuracy Levels 100% - 2:**
Weighed and transferred 0.6338 g of placebo, 175.15 mg of Lopinavir drug, 43.56 mg of Ritonavir drug in 250 milliliter of flask to it added 150 milliliter of Methanol. Sonicated for 30 Minutes, diluted to final volume of flask by Methyl alcohol and mixed properly, By filtration of Sample from Zero point Forty five (0.4 micro meter) Micron Filters discarded first 3.0 milliliter solution and Again diluted to 50 milliliter volumetric flask with mobile phase having 5 milliliter of this Sample (stock Sample).

**Making of Recovery ie.accuracy Levels 100% - 3:**
Weighed and transferred 0.6345 g of placebo, 175.22 mg of Lopinavir drug, 43.35 mg of Ritonavir drug in 250 milliliter of flask to it added 150 milliliter of Methanol. Sonicated for 30 Minutes, diluted to final volume of flask by Methyl alcohol and mixed properly, By filtration of Sample from Zero point Forty five (0.4 micro meter) Micron Filters discarded first 3.0 milliliter solution and Again diluted to 50 milliliter volumetric flask with mobile phase having 5 milliliter of this Sample (stock Sample).

**Making of Recovery ie.accuracy Levels 150% - 1:**
Weighed and transferred 0.6352 g of placebo, 262.31 mg of Lopinavir drug, 67.53 mg of Ritonavir drug in 250 milliliter of flask to it added 150 milliliter of Methanol. Sonicated for 30 Minutes, diluted to final volume of flask by Methyl alcohol and
mixed properly. By filtration of Sample from Zero point Forty five (0.4 micro meter) Micron Filters discarded first 3.0 milliliter solution and Again diluted to 50 milliliter volumetric flask with mobile phase having 5 milliliter of this Sample (stock Sample).

**Making of Recovery ie.accuracy Levels 150% - 2:**
Weighed and transferred 0.6314 g of placebo, 262.54 mg of Lopinavir drug, 67.52 Ritonavir drug in 250 milliliter of flask to it added 150 milliliter of Methanol Sonicated for 30 Minutes, diluted to final volume of flask by Methyl alcohol and mixed properly, By filtration of Sample from Zero point Forty five (0.4 micro meter) Micron Filters discarded first 3.0 milliliter solution and Again diluted to 50 milliliter volumetric flask with mobile phase having 5 milliliter of this Sample (stock Sample).

**Making of Recovery ie.accuracy Levels 150% - 3:**
Weighed and transferred 0.6344 g of placebo, 262.31 mg of Lopinavir drug, 67.56 Ritonavir drug in 250 milliliter of flask to it added 150 milliliter of Methanol Sonicated for 30 Minutes, diluted to final volume of flask by Methyl alcohol and mixed properly, By filtration of Sample from Zero point Forty five (0.4 micro meter) Micron Filters discarded first 3.0 milliliter solution and Again diluted to 50 milliliter volumetric flask with mobile phase having 5 milliliter of this Sample (stock Sample).

The sequence of injections for Recovery ie.accuracy is given as

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>No. of Injections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank Sample</td>
<td>1</td>
</tr>
<tr>
<td>Standard Sample (Reference</td>
<td>5</td>
</tr>
<tr>
<td>Sample)</td>
<td></td>
</tr>
<tr>
<td>Rec-50% / 1</td>
<td>1</td>
</tr>
<tr>
<td>Rec-50% / 2</td>
<td>1</td>
</tr>
<tr>
<td>Rec-50% / 3</td>
<td>1</td>
</tr>
<tr>
<td>Rec-100% / 1</td>
<td>1</td>
</tr>
<tr>
<td>Rec-100% / 2</td>
<td>1</td>
</tr>
<tr>
<td>Rec-100% / 3</td>
<td>1</td>
</tr>
</tbody>
</table>
Rec-150% / 1  1
Rec-150% / 2  1
Rec-150% / 3  1
Bracketing standard_1  1

Calculation:

Recovery ie. accuracy (% Recovery) = \(\frac{\text{Amount found}}{\text{Amount added}} \times 100\)

Data evaluation:

Calculated percent recovery for all level and reported along with percent mean recovery for all level.
6. Filter Validation:

Experiment:

**Buffer Preparation: (solution A)**

Dissolved 34.210 g Dihydrogen Potassium phosphate in to 5 liter water. Make pH for this buffer Sample to 3.01 ± 0.05 Unit with help of OPA (O-phosphoric Acid) and filter this from Zero point Forty five (0.4 micro meter) um nylon filters.

**Making of Dilute Orthophosphoric acid Sample:**

Take 200 milliliter of volumetric flask added 20 milliliter of OPA (O-phosphoric Acid) and diluted this flask to its ring mark and homogeneously mixed.

**Making of Solvent Mixture Sample:**

Prepared mixture of ACN (Acetonitrile) and Methanol in composition of 4000:1000 milliliter.

**Mobile Phase:**

Mixed a The Mixture of filtered solution A (Degassed) and Solvent Mixture in proportion of 2250:2750.

**Diluent Sample:**

I. Methanol
II. Mobile Phase.

**System Controlling Parameter**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name of system</td>
<td>HPLC</td>
</tr>
<tr>
<td>Used Column</td>
<td>SB C-18, 50 millimetre x 4.6 millimetre, 3.5 µm. (Zorbax)</td>
</tr>
<tr>
<td>System flow- rate</td>
<td>1.0 milliliter / minute</td>
</tr>
<tr>
<td>Absorption Wavelength</td>
<td>210 Nano-meter</td>
</tr>
<tr>
<td>Temperature of Column oven</td>
<td>25°C</td>
</tr>
<tr>
<td>Volume at time of injection</td>
<td>10 µl</td>
</tr>
<tr>
<td>Run time of injection</td>
<td>15 Minutes</td>
</tr>
<tr>
<td>Mode type For Analysis</td>
<td>Isocratic</td>
</tr>
</tbody>
</table>
Making of Lopinavir Stock Sample:
Transferred 35.45 mg of Lopinavir standard in 50 milliliter of flask to it added 35 milli liter of Methanol dissolved and make up it to volume (ring mark) of flask by Methyl alcohol (Methanol).

Making of Ritonavir Stock Sample:
Transferred 35.05 mg of Ritonavir standard in 200 milli liter of flask to it added 150 milli liter of Methanol dissolved and make up it to volume (ring mark) of flask by Methyl alcohol (Methanol).

Making of Standard Sample (Reference Sample):
Added 5 ml each of stock Sample to 50.0 mili liter flask (Volumetric) and diluted to 50 mili liter it to volume (ring mark) of flask using diluent i.e Mobile-phase

Making of sample filtered and Unfiltered Sample:
Transferred 1.0050 g of Capsules Content in 250 milli liter of flask. Added 170 ml of Methanol Sonicated it through occasional Slight Shaking for 30 Minutes diluted to final volume of flask with help of Methyl alcohol (Methanol solvent) & homogeneously mixed. Centrifuge half part this Sample at 2000 rpm for 5 minutes. Decanted the supernatant Sample in another test tube. Injected this Sample as unfiltered test Sample. From the remaining half part of the Sample, filter the Sample through 5 different (same make) Zero point Forty five (0.4 micro meter) micron filter. Discard first 3.0 millilitre solution and again diluted 5 milliliter of respective soluion in 50 milliliter by mobile phase; fill five vials of this Sample.
The sequence of injections for filter validation is given as:

An example of sequence for filter validation

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>No. of Injections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank Sample</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Standard Sample (Reference Sample)</td>
<td>Replicates 5</td>
</tr>
<tr>
<td>Unfiltered Test Sol.</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Filtered Test Sol. – 1</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Filtered Test Sol. – 2</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Filtered Test Sol. – 3</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Filtered Test Sol. – 4</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Filtered Test Sol. – 5</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Check standard</td>
<td>Replicate 1</td>
</tr>
</tbody>
</table>

Data evaluation:
The % assay of Lopinavir & Ritonavir filtered, unfiltered test Sample will be calculated and reported along with the difference between unfiltered and filtered test Sample.
7. Robustness:

Experiment: Low Flow

Buffer Preparation: (solution A)
Dissolved 35.0025 g Dihydrogen Potassium phosphate in to 5 liter water. Make pH for this buffer Sample to 3.00 ± 0.05 Unit with help of OPA (O-phosphoric Acid) and filter this from Zero point Forty five (0.4 micro meter) um nylon filters.

Making of Dilute Orthophosphoric acid Sample:
Take 200 milliliter of volumetric flask added 20 milliliter of OPA (O-phosphoric Acid) and diluted this flask to its ring mark and homogeneously mixed

Making of Solvent Mixture Sample:
Prepared mixture of ACN (Acetonitrile) and Methanol in composition of 4000:1000 milliliter

Mobile Phase:
Mixed a The Mixture of filtered solution A (Degassed) and Solvent Mixture in proportion of 2250:2750.

Diluent Sample:

I. Methanol
II. Mobile Phase.

System Controlling Parameter
Name of system : HPLC
Used Column : SB C-18, 50 millimetre x 4.6 millimetre, 3.5 µm. (Zorbax)
System flow- rate : 0.9 milliliter / minute (Actual 1.0 ml)
Absorption Wavelength : 210 Nano-meter
Temperature of Column oven : 25°C
Volume at time of injection : 10 µl
Run time of injection : 15 Minutes
Mode type For Analysis : Isocratic

78
Making of Lopinavir Stock Sample:
Transferred 35.09 mg of Lopinavir standard in 50 milliliter of flask to it added 35 milliliter of Methanol dissolved and make up it to volume (ring mark) of flask by Methyl alcohol (Methanol).

Making of Ritonavir Stock Sample:
Transferred 35.46 mg of Ritonavir standard in 200 millili liter of flask to it added 150 millili liter of Methanol dissolved and make up it to volume (ring mark) of flask by Methyl alcohol (Methanol).

Making of Standard Sample (Reference Sample) :
Added 5 ml each of stock Sample to 50.0 milli liter flask (Volumetric) and diluted to 50 millili liter it to volume (ring mark) of flask using diluent i.e Mobile-phase

Making of sample:
Transferred 1.0112 g of Capsules Content in 250 millili liter of flask. Added 170 ml of Methanol Sonicated it through occasional Slight Shaking for 30 Minutes diluted to final volume of flask by Methyl alcohol and mixed properly, By filtration of Sample from Zero point Forty five (0.4 micro meter) Micron size filters discarded first 3.0 milliliter solution and Again diluted to 50 milliliter volumetric flask with mobile phase having 5 milliliter of this Sample (stock Sample).

The sequence of injections for Robustness Low Flow given as:

Sequence for Robustness Low Flow

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>No. of Injections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank Sample</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Standard Sample (Reference Sample)</td>
<td>Replicate 5</td>
</tr>
<tr>
<td>Test sample</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Check standard</td>
<td>Replicate 1</td>
</tr>
</tbody>
</table>
8. Experiment: Robustness-High Flow

Experiment: Low Flow

Buffer Preparation: (solution A)
Dissolved 35.117 g Dihydrogen Potassium phosphate in to 5 liter water. Make pH for this buffer Sample to 3.00 ± 0.05 Unit with help of OPA (O-phosphoric Acid) and filter this from Zero point Forty five (0.4 micro meter) um nylon filters.

Making of Dilute Orthophosphoric acid Sample:
Take 200 milliliter of volumetric flask added 20 milliliter of OPA (O-phosphoric Acid) and diluted this flask to its ring mark and homogeneously mixed

Making of Solvent Mixture Sample:
Prepared mixture of ACN (Acetonitrile) and Methanol in composition of 4000:1000 milliliter

Mobile Phase:
Mixed a The Mixture of filtered solution A (Degassed) and Solvent Mixture in proportion of 2250:2750.

Diluent Sample:
  I. Methanol
  II. Mobile Phase.

System Controlling Parameter
Name of system : HPLC
Used Column : SB C-18, 50 millimetre x 4.6 millimetre, 3.5 μm. (Zorbax)
System flow- rate : 1.1 milliliter / minute (Actual 1.0 ml)
Absorption Wavelength : 210 Nano-meter
Temperature of Column oven : 25°C
Volume at time of injection : 10 μl
Run time of injection : 15 Minutes
Mode type For Analysis : Isocratic
Making of Lopinavir Stock Sample:
Transferred 34.99 mg of Lopinavir standard in 50 milliliter of flask to it added 35 milliliter of Methanol dissolved and make up it to volume (ring mark) of flask by Methyl alcohol (Methanol).

Making of Ritonavir Stock Sample:
Transferred 34.96 mg of Ritonavir standard in 200 milliliter of flask to it added 150 milliliter of Methanol dissolved and make up it to volume (ring mark) of flask by Methyl alcohol (Methanol).

Making of Standard Sample (Reference Sample):
Added 5 ml each of stock Sample to 50.0 milliliter flask (Volumetric) and diluted to 50 milliliter it to volume (ring mark) of flask using diluent i,e Mobile-phase.

Making of sample:
Transferred 0.9998 g of Capsules Content in 250 milliliter of flask. Added 170 ml of Methanol Sonicated it through occasional Slight Shaking for 30 Minutes diluted to final volume of flask by Methyl alcohol and mixed properly. By filtration of Sample from Zero point Forty five (0.4 micro meter) Micron size filters discarded first 3.0 milliliter solution and Again diluted to 50 milliliter volumetric flask with mobile phase having 5 milliliter of this Sample (stock Sample).

The sequence of injections for Robustness high Flow given as:

**Sequence for Robustness high Flow**

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>No. of Injections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank Sample</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Standard Sample (Reference Sample)</td>
<td>Replicate 5</td>
</tr>
<tr>
<td>Test sample</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Check standard</td>
<td>Replicate 1</td>
</tr>
</tbody>
</table>
Data Evaluation
Recorded each chromatogram. All the injections will be processed at the wavelength provided in the method and Calculated % RSD for Replicate injection of Lopinavir & Ritonavir peak.
Experiment: Low pH - 2.8

Buffer Preparation: (solution A)
Dissolved 35.105 g Dihydrogen Potassium phosphate in to 5 liter water. Make pH for this buffer Sample to $2.82 \pm 0.05$ Unit with help of OPA (O-phosphoric Acid) and filter this from Zero point Forty five (0.4 micro meter) um nylon filters.

(Actual pH = 3.0)

Making of Dilute Orthophosphoric acid Sample:
Take 200 milliliter of volumetric flask added 20 milliliter of OPA (O-phosphoric Acid) and diluted this flask to its ring mark and homogeneously mixed

Making of Solvent Mixture Sample:
Prepared mixture of ACN (Acetonitrile) and Methanol in composition of 4000:1000 milliliter

Mobile Phase:
Mixed a The Mixture of filtered solution A (Degassed) and Solvent Mixture in proportion of 2250:2750.

Diluent Sample:
I. Methanol
II. Mobile Phase.

System Controlling Parameter
Name of system : HPLC
Used Column : SB C-18, 50 millimetre x 4.6 millimetre, 3.5 µm. (Zorbax)
System flow- rate : 1.0 milliliter / minute
Absorption Wavelength : 210 Nano-meter
Temperature of Column oven : 25°C
Volume at time of injection : 10 µl
Run time of injection : 15 Minutes
Mode type For Analysis : Isocratic
Making of Lopinavir Stock Sample:
Transferred 35.09 mg of Lopinavir standard in 50 milliliter of flask to it added 35 milliliter of Methanol dissolved and make up it to volume (ring mark) of flask by Methyl alcohol (Methanol).

Making of Ritonavir Stock Sample:
Transferred 35.16 mg of Ritonavir standard in 200 milliliter of flask to it added 150 milliliter of Methanol dissolved and make up it to volume (ring mark) of flask by Methyl alcohol (Methanol).

Making of Standard Sample (Reference Sample) :
Added 5 ml each of stock Sample to 50.0 milliliter flask (Volumetric) and diluted to 50 milliliter it to volume (ring mark) of flask using diluent i.e Mobile-phase

Making of sample:
Transferred 0.9991 g of Capsules Content in 250 milliliter of flask. Added 170 ml of Methanol Sonicated it through occasional Slight Shaking for 30 Minutes diluted to final volume of flask by Methyl alcohol and mixed properly, By filtration of Sample from Zero point Forty five (0.4 micro meter) Micron size filters discarded first 3.0 milliliter solution and Again diluted to 50 milliliter volumetric flask with mobile phase having 5 milliliter of this Sample (stock Sample).

The sequence of injections for Robustness Low pH given as:

**Sequence for Robustness Low pH**

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>No. of Injections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank Sample</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Standard Sample (Reference Sample)</td>
<td>Replicate 5</td>
</tr>
<tr>
<td>Test sample Low pH</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Check standard</td>
<td>Replicate 1</td>
</tr>
</tbody>
</table>
**Data Evaluation**

Recorded each chromatogram. All the injections will be processed at the wavelength provided in the method and Calculated % RSD for Replicate injection of Lopinavir & Ritonavir peak.
10. Experiment: High pH – 3.2

Buffer Preparation: (solution A)

Dissolved 35.005 g Dihydrogen Potassium phosphate in to 5 liter water. Make pH for this buffer Sample to 3.21 ± 0.05 Unit with help of OPA (O-phosphoric Acid) and filter this from Zero point Forty five (0.4 micro meter) um nylon filters. (Actual pH = 3.0)

Making of Dilute Orthophosphoric acid Sample:

Take 200 milliliter of volumetric flask added 20 milliliter of OPA (O-phosphoric Acid) and diluted this flask to its ring mark and homogeneously mixed.

Making of Solvent Mixture Sample:

Prepared mixture of ACN (Acetonitrile) and Methanol in composition of 4000:1000 milliliter

Mobile Phase:

Mixed a The Mixture of filtered solution A (Degassed) and Solvent Mixture in proportion of 2250:2750.

Diluent Sample:

I. Methanol
II. Mobile Phase.

System Controlling Parameter

Name of system : HPLC
Used Column : SB C-18, 50 millimetre x 4.6 millimetre, 3.5 µm. (Zorbax)
System flow-rate : 1.0 milliliter / minute
Absorption Wavelength : 210 Nano-meter
Temperature of Column oven : 25°C
Volume at time of injection : 10 µl
Run time of injection : 15 Minutes
Mode type For Analysis : Isocratic
Making of Lopinavir Stock Sample:
Transferred 35.46 mg of Lopinavir standard in 50 milliliter of flask to it added 35 milliliter of Methanol dissolved and make up it to volume (ring mark) of flask by Methyl alcohol (Methanol).

Making of Ritonavir Stock Sample:
Transferred 35.12 mg of Ritonavir standard in 200 milliliter of flask to it added 150 milliliter of Methanol dissolved and make up it to volume (ring mark) of flask by Methyl alcohol (Methanol).

Making of Standard Sample (Reference Sample):
Added 5 ml each of stock Sample to 50.0 milliliter flask (Volumetric) and diluted to 50 milliliter it to volume (ring mark) of flask using diluent i.e Mobile-phase

Making of sample:
Transferred 1.0114 g of Capsules Content in 250 milliliter of flask. Added 170 ml of Methanol Sonicated it through occasional Slight Shaking for 30 Minutes diluted to final volume of flask by Methyl alcohol and mixed properly, By filtration of Sample from Zero point Forty five (0.4 micro meter) Micron size filters discarded first 3.0 milliliter solution and Again diluted to 50 milliliter volumetric flask with mobile phase having 5 milliliter of this Sample (stock Sample).

Data Evaluation
Recorded each chromatogram. All the injections will be processed at the wavelength provided in the method and Calculated % RSD for Replicate injection of Lopinavir & Ritonavir peak.
11. Sample Stability:

Experiment:

Buffer Preparation: (solution A)
Dissolved 34.987 g Dihydrogen Potassium phosphate in to 5 liter water. Make pH for this buffer Sample to $3.03 \pm 0.05$ Unit with help of OPA (O-phosphoric Acid) and filter this from Zero point Forty five (0.4 micro meter) um nylon filters.

Making of Dilute Orthophosphoric acid Sample:
Take 200 milliliter of volumetric flask added 20 milliliter of OPA (O-phosphoric Acid) and diluted this flask to its ring mark and homogeneously mixed

Making of Solvent Mixture Sample:
Prepared mixture of ACN (Acetonitrile) and Methanol in composition of 4000:1000 milliliter

Mobile Phase:
Mixed a The Mixture of filtered solution A (Degassed) and Solvent Mixture in proportion of 2250:2750.

Diluent Sample:

I. Methanol
II. Mobile Phase.

System Controlling Parameter
Name of system : HPLC
Used Column : SB C-18, 50 millimetre x 4.6 millimetre, 3.5 µm. (Zorbax)
System flow-rate : 1.0 milliliter / minute
Absorption Wavelength : 210 Nano-meter
Temperature of Column oven : 25°C
Volume at time of injection : 10 µl
Run time of injection : 15 Minutes
Mode type For Analysis : Isocratic
Making of Lopinavir Stock Sample:
Transferred 35.01 mg of Lopinavir standard in 50 milliliter of flask to it added 35 milliliter of Methanol dissolved and make up it to volume (ring mark) of flask by Methyl alcohol (Methanol).

Making of Ritonavir Stock Sample:
Transferred 35.04 mg of Ritonavir standard in 200 milliliter of flask to it added 150 milliliter of Methanol dissolved and make up it to volume (ring mark) of flask by Methyl alcohol (Methanol).

Making of Standard Sample (Reference Sample) :
Added 5 ml each of stock Sample to 50.0 milliliter flask (Volumetric) and diluted to 50 milliliter it to volume (ring mark) of flask using diluent i.e Mobile-phase

Making of sample:
Transferred 1.0214 g of Capsules Content in 250 milliliter of flask. Added 170 ml of Methanol Sonicated it through occasional Slight Shaking for 30 Minutes diluted to final volume of flask by Methyl alcohol and mixed properly, By filtration of Sample from Zero point Forty five (0.4 micro meter) Micron size filters discarded first 3.0 milliliter solution and Again diluted to 50 milliliter volumetric flask with mobile phase having 5 milliliter of this Sample (stock Sample).

The sequence of injections for Sample stability is given as:
<table>
<thead>
<tr>
<th>Sample Name</th>
<th>No. of Injections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank Sample</td>
<td>1</td>
</tr>
<tr>
<td>Freshly prepared standard Sample (Reference Sample)</td>
<td>5</td>
</tr>
<tr>
<td>Standard Sample (Reference Sample) – After 2 hours</td>
<td>1</td>
</tr>
<tr>
<td>Sample – After 2 hours</td>
<td>1</td>
</tr>
<tr>
<td>Standard Sample (Reference Sample) – After 3 hours</td>
<td>1</td>
</tr>
<tr>
<td>Sample – After 3 hours</td>
<td>1</td>
</tr>
<tr>
<td>Standard Sample (Reference Sample) – After 4 hours</td>
<td>1</td>
</tr>
<tr>
<td>Sample – After 4 hours</td>
<td>1</td>
</tr>
<tr>
<td>Standard Sample (Reference Sample) – After 8 hours</td>
<td>1</td>
</tr>
<tr>
<td>Sample – After 8 hours</td>
<td>1</td>
</tr>
<tr>
<td>Standard Sample (Reference Sample) – After 12 hours</td>
<td>1</td>
</tr>
<tr>
<td>Sample – After 12 hours</td>
<td>1</td>
</tr>
<tr>
<td>Standard Sample (Reference Sample) – After 16 hours</td>
<td>1</td>
</tr>
<tr>
<td>Sample – After 16 hours</td>
<td>1</td>
</tr>
<tr>
<td>Standard Sample (Reference Sample) – After 20 hours</td>
<td>1</td>
</tr>
<tr>
<td>Sample – After 20 hours</td>
<td>1</td>
</tr>
<tr>
<td>Standard Sample (Reference Sample) – After 24 hours</td>
<td>1</td>
</tr>
<tr>
<td>Sample – After 24 hours</td>
<td>1</td>
</tr>
<tr>
<td>Bracketing Standard</td>
<td>1</td>
</tr>
</tbody>
</table>
Data evaluation:
% RSD of the results of stored test Sample compared to the result of freshly prepared test Sample for Lopinavir & Ritonavir Soft gelatin Capsules. Calculated the % recovery.
6.1.0. EXPERIMENTAL PLAN AND DATA EVALUATION FOR MONTAIR TABLETS

Specificity:

Selectivity – Experiment:
Prepared the Diluent blank Sample as per the analytical method developed.

Making of Montelukast sodium Standard Sample:
Weighed accurately 25.96 milligram working standard (Montelukast sodium) and transferred it to 50 milliliter of flask to it added 30 milliliter of diluent. To this flask Sonicated for 5 minutes with shaking dilute to ring mark of flask volume. (Stock-1) Dilute 10 milliliter of respective solution (Stock-1) to 50 milliliter of flask up to its mark with diluent.

Standard Sample (Reference Sample):
Used Montelukast sodium standard Sample preparation as standard Sample (Reference Sample).

Test Sample Preparation:
Take 100 milliliter of flask and to this transferred tablets fine powder equal volume to 10 milligrams of montelukast. To the flask added 75 milliliter of diluent solution, shake by means of mechanically to dispersed the tablets powder. Sonicated this to dissolve and dilute to flask mark and filtered it from Zero point Forty five (0.4 micro meter) micron filter paper.

Making of Excipient blend Sample:
Take 100 milliliter of flask and to this transferred 390.12 miligram of excipient blend. To the flask added 75 milliliter of diluent solution, shake by means of mechanically to disperse the tablets powder. Sonicated this to dissolve and dilute to flask mark and filtered it from Zero point Forty five (0.4 micro meter) micron filter paper.
Making of Sulphoxide Impurity Stock Sample:
Take 20 milliliter of flask and to this transferred 2.124 milligram of Sulphoxide Impurity. To the flask added 15 milliliter of diluent solution, shake by means of mechanically & sonicated this to dissolve and made up to flask mark.

Making of Sulphoxide Impurity Sample:
Transferred 2 milliliter of Sulphoxide impurity stock Sample to 200 milliliter of flask and dilute it with diluent and mixed.

Test Sample Preparation spiked with known impurities:
Take 100 milliliter of flask and to this transferred tablets fine powder equal volume to 10 milligrams of montelukast. To the flask added 75 milliliter of diluent solution, shake by means of mechanically to dispersed the tablets powder. Sonicated this to dissolve added 1.0 milliliter of Sulphoxide impurity stock Sample and dilute to flask mark and filtered it from Zero point Forty five (0.4 micro meter) micron filter paper.
The sequence of samples for selectivity given in as:

An example of sequence for selectivity

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>No. of Injections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluent Blank Sample</td>
<td>2</td>
</tr>
<tr>
<td>Standard Sample (Reference Sample)</td>
<td>5</td>
</tr>
<tr>
<td>Diluent Blank Sample</td>
<td>1</td>
</tr>
<tr>
<td>Excipient blend Sample</td>
<td>1</td>
</tr>
<tr>
<td>Sulphoxide Impurity Sample</td>
<td>1</td>
</tr>
<tr>
<td>Test Sample</td>
<td>1</td>
</tr>
<tr>
<td>Test Sample spiked with Impurities</td>
<td>1</td>
</tr>
<tr>
<td>Bracketing standard</td>
<td>2</td>
</tr>
</tbody>
</table>
Data evaluation:
Record each chromatogram. All the injections will be processed at the wavelength provided in the method and selectivity will be demonstrated with regards to non-interference from peaks due to Diluent blank Sample, excipient blend Sample and known impurities with Montelukast peak. The peak purity will be determined for Montelukast peak.
Forced degradation:

Experiment:
Degradating agents will be added separately to Excipient blend and Montelukast Sodium Chewable Tablets 5 mg. Degrading agents will be 1N HCl (Acid degradation), 1N NaOH (Base degradation), 30% Hydrogen peroxide (Oxidative degradation), Heat Sample (60°C), Heat solid (80°C), Humidity (75% RH) and Photolysis.

Making of Samples for forced degradation studies   (Excipient blend)

Untreated sample:
Take 100 milliliter of flask and to this transferred 390.12 milligram of excipient blend. To the flask added 75 milliliter of diluent solution, shake by means of mechanically to disperse the tablets powder. Sonicated this to dissolve and dilute to flask mark and filtered it from Zero point Forty five (0.4 micro meter) micron filter paper.

Acid degradation:  (1 N HCl)
Take 100 milliliter of flask and to this transferred 390.12 milligram of excipient blend. To the flask added 75 milliliter of diluent solution, shake by means of mechanically to disperse the tablets powder. Sonicated this to dissolve, added 2.0 milliliter of 1N HCl to the flask, Kept the flask for 2 hours at room temperature. Neutralized the Sample with 1N NaOH and dilute to flask mark and filtered it from Zero point Forty five (0.4 micro meter) micron filter paper.
A blank Sample (without excipient blend) prepared in similar way.

Base degradation: (1 N NaOH)
Take 100 milliliter of flask and to this transferred 390.12 milligram of excipient blend. To the flask added 75 milliliter of diluent solution, shake by means of mechanically to disperse the tablets powder. Sonicated this to dissolve, added 2.0 milliliter of 1N NaOH to the flask, Kept the flask for 2 hours at room temperature. Neutralized the Sample with 1N HCl and dilute to flask mark and filtered it from Zero point Forty five (0.4 micro meter) micron filter paper.
A blank Sample (without excipient blend) prepared in similar way.
Peroxide dégradations: 30% H$_2$O$_2$

Take 100 milliliter of flask and to this transferred 390.12 milligram of excipient blend. To the flask added 75 milliliter of diluent solution, shake by means of mechanically to disperse the tablets powder. Sonicated this to dissolve, 2.0 milliliter 30% Hydrogen peroxide to the flask and mixed well Kept it 10 minutes and dilute to flask mark and filtered it from Zero point Forty five (0.4 micro meter) micron filter paper.

A blank Sample (without excipient blend) prepared in similar way.

In same way prepared sample for tablets and API by giving same treatment and run sequence as follow:

### An example of Sequence for Forced degradation

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>No. of Injections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluent Blank</td>
<td>Replicates 1</td>
</tr>
<tr>
<td>Suitability standard</td>
<td>Replicates 5</td>
</tr>
<tr>
<td>Blank-Untreated</td>
<td>Replicates 1</td>
</tr>
<tr>
<td>Blank- acid degradation</td>
<td>Replicates 1</td>
</tr>
<tr>
<td>Blank- base degradation</td>
<td>Replicates 1</td>
</tr>
<tr>
<td>Blank- peroxide degradation</td>
<td>Replicates 1</td>
</tr>
<tr>
<td>Placebo -Untreated</td>
<td>Replicates 1</td>
</tr>
<tr>
<td>Placebo - acid degradation</td>
<td>Replicates 1</td>
</tr>
<tr>
<td>Placebo - base degradation</td>
<td>Replicates 1</td>
</tr>
<tr>
<td>Placebo - peroxide degradation</td>
<td>Replicates 1</td>
</tr>
<tr>
<td>Tablets sample -Untreated</td>
<td>Replicates 1</td>
</tr>
<tr>
<td>Tablets sample - acid degradation</td>
<td>Replicates 1</td>
</tr>
<tr>
<td>Check standard</td>
<td>Replicates 1</td>
</tr>
<tr>
<td>Tablets sample - base degradation</td>
<td>Replicates 1</td>
</tr>
<tr>
<td>Tablets sample- peroxide degradation</td>
<td>Replicates 1</td>
</tr>
<tr>
<td>Check standard</td>
<td>Replicates 1</td>
</tr>
</tbody>
</table>
**Data evaluation:**

Plot a linearity graph of average area at each level against the concentration (ppm) and determine the correlation coefficient.
Precision:

System Precision:

Experiment:

Remaining preparation as per analytical method.

Making of Montelukast sodium Standard Sample:
Weighed accurately 25.96 milligram working standard (Montelukast sodium) and transferred it to 50 milliliter of flask to it added 30 milliliter of diluent. To this flask Sonicated for 5 minutes with shaking dilute to ring mark of flask volume. (Stock-1) Dilute 10 milliliter of respective solution (Stock-1) to 50 milliliter of flask up to its mark with diluent.

Standard Sample (Reference Sample):
Used Montelukast sodium standard Sample preparation as standard Sample (Reference Sample).

Make injections of standard Sample (Reference Sample) into the HPLC using the method of analysis. The sequence of injections for system precision is given as:

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>No. of Injections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluent Blank Sample</td>
<td>Replicate 2</td>
</tr>
<tr>
<td>Standard Sample (Reference Sample)</td>
<td>Replicate - 1</td>
</tr>
<tr>
<td>Standard Sample (Reference Sample)</td>
<td>Replicate – 1</td>
</tr>
<tr>
<td>Standard Sample (Reference Sample)</td>
<td>Replicate – 1</td>
</tr>
<tr>
<td>Standard Sample (Reference Sample)</td>
<td>Replicate – 1</td>
</tr>
<tr>
<td>Standard Sample (Reference Sample)</td>
<td>Replicate - 1</td>
</tr>
</tbody>
</table>
Data evaluation:
Calculate and report SD and % RSD of the five replicate injections of standard Sample (Reference Sample).
Percent RSD of Area observed for Montelukast from five reference sample does not exceed more than 2.00 %.

Method Precision:
Experiment:
Prepared six test Samples of Montelukast Sodium Chewable tablets 5 mg as per analytical method and inject into the HPLC as per analytical method.

Making of Montelukast sodium Standard Sample:
Weighed accurately 25.96 milligram working standard (Montelukast sodium) and transferred it to 50 milliliter of flask to it added 30 milliliter of diluent. To this flask Sonicated for 5 minutes with shaking dilute to ring mark of flask volume. (Stock-1) Dilute 10 milliliter of respective solution (Stock-1) to 50 milliliter of flask up to its mark with diluent.

Standard Sample (Reference Sample) :
Used Montelukast sodium standard Sample preparation as standard Sample (Reference Sample).

Test Sample Preparation:
Take 100 milliliter of flask and to this transferred tablets fine powder equal volume to 10 milligrams of montelukast. To the flask added 75 milliliter of diluent solution, shake by means of mechanically to disperse the tablets powder. Sonicated this to dissolve and dilute to flask mark and filtered it from Zero point Forty five (0.4 micron) micron filter paper.

The sequence of injections for method precision is given as:
### An example of Sequence for Method Precision

<table>
<thead>
<tr>
<th>Test solution Name</th>
<th>Injections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluent Blank Sample</td>
<td>Replicate 2</td>
</tr>
<tr>
<td>Standard Sample (Reference Sample)</td>
<td>Replicates 5</td>
</tr>
<tr>
<td>Test solution – I</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Test solution – II</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Test solution – III</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Test solution – IV</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Test solution – V</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Test solution – VI</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Check standard</td>
<td>Replicate 2</td>
</tr>
</tbody>
</table>

### Data evaluation:
The % assay will be calculated and reported along with the SD and % RSD of the six test Samples. Percent RSD not exceed than 2.00
**Recovery or accuracy (% Recovery):**

**Experiment:**

Weighed and transferred accurately, in triplicate at each level, about 390 mg of excipient blend and add to it Montelukast Sodium API at 50%, 100% and 150% of the working concentration. Working (assay) concentration of Montelukast is 100 ppm. Prepare the Samples as per the analytical method.

Inject each of the samples and take area count for calculations.

**Table 10: Dilutions for Recovery ie. accuracy for Montelukast.**

<table>
<thead>
<tr>
<th>Level</th>
<th>Sample Name</th>
<th>Amount of Montelukast Sodium API to be weighed (mg)</th>
<th>Make up volume in mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>First Level</td>
<td>Rec-50% / 1</td>
<td>5.2</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Rec-50% / 2</td>
<td>5.2</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Rec-50% / 3</td>
<td>5.2</td>
<td>100</td>
</tr>
<tr>
<td>Second Level</td>
<td>Rec-100% / 1</td>
<td>10.4</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Rec-100% / 2</td>
<td>10.4</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Rec-100% / 3</td>
<td>10.4</td>
<td>100</td>
</tr>
<tr>
<td>Third Level</td>
<td>Rec-150% / 1</td>
<td>15.6</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Rec-150% / 2</td>
<td>15.6</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Rec-150% / 3</td>
<td>15.6</td>
<td>100</td>
</tr>
</tbody>
</table>

The sequence of injections for Recovery ie. accuracy is given as:
An example of sequence for Recovery ie. accuracy

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>No. of Injections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluent Blank Sample</td>
<td>2</td>
</tr>
<tr>
<td>Standard Sample (Reference Sample)</td>
<td>5</td>
</tr>
<tr>
<td>Rec-50% / 1</td>
<td>1</td>
</tr>
<tr>
<td>Rec-50% / 2</td>
<td>1</td>
</tr>
<tr>
<td>Rec-50% / 3</td>
<td>1</td>
</tr>
<tr>
<td>Rec-100% / 1</td>
<td>1</td>
</tr>
<tr>
<td>Rec-100% / 2</td>
<td>1</td>
</tr>
<tr>
<td>Rec-100% / 3</td>
<td>1</td>
</tr>
<tr>
<td>Rec-150% / 1</td>
<td>1</td>
</tr>
<tr>
<td>Rec-150% / 2</td>
<td>1</td>
</tr>
<tr>
<td>Rec-150% / 3</td>
<td>1</td>
</tr>
<tr>
<td>Bracketing standard_1</td>
<td>2</td>
</tr>
</tbody>
</table>

**Calculation:**

Recovery ie. accuracy (% Recovery) = \( \frac{\text{Amount found}}{\text{Amount added}} \times 100 \)

**Data evaluation:**

Calculate percent recovery all level and report along with percent mean recovery all level.
Filter Validation:

Experiment:
Take 100 milliliter of flask and to this transferred tablets fine powder equal volume to 10 milligrams of montelukast. To the flask added 75 milliliter of diluent solution, shake by means of mechanically to dispersed the tablets powder. Sonicated this to dissolve and dilute to flask mark and filtered it from Zero point Forty five (0.4 micro meter) micron filter paper.
From above take 50 milliliter of sample centrifuge this Sample at about 6000 rpm for 25 min, take supernatant Sample in other flask or vial, Inject this Sample as unfiltered test Sample. From the remaining half part of the Sample, filter the Sample through 5 different (same make) Zero point Forty five (0.4 micro meter) micron filter. Discard first 3.0 millilitre solutions. Filled five vials of this Sample.
Use this as filtered test Sample. Analyze all filtered, unfiltered Sample in single sequence.

The sequence of injections for filter validation is given as:

An example of sequence for filter validation

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>No. of Injections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluent Blank Sample</td>
<td>Replicates 2</td>
</tr>
<tr>
<td>Standard Sample (Reference Sample)</td>
<td>Replicates 5</td>
</tr>
<tr>
<td>Unfiltered Test Sample</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Test Sample filtered – 1</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Test Sample filtered – 2</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Test Sample filtered – 3</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Test Sample filtered – 4</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Test Sample filtered – 5</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Check standard</td>
<td>Replicate 2</td>
</tr>
</tbody>
</table>
Data evaluation:
The % assay of Montelukast in Montelukast Sodium Chewable Tablets 5 mg with filtered, unfiltered test Sample will be calculated and reported along with the difference between unfiltered and filtered test Sample.
Robustness:
Experiment:
Prepare two test Samples of the same lot (as used in 3.2 and 3.3) of Montelukast Sodium Chewable Tablets 5 mg as per analytical method and inject along with the Diluent blank Sample and standard Sample (Reference Sample) using different chromatographic conditions as shown below:

Column temp: Low temp and high temp changed (± 5°C)

pH changed for buffer: Low pH buffer and high pH Buffer (± 0.2 Unit)

Different Wavelength: Low and high wave length (± 2 Nanometer)

Column compartment Temp:
(Normal Experimental Condition: 30°C)

An example of sequence for change in Column oven temperature (25°C)

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>No. of Injections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluent Blank Sample</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Standard Sample (Reference Sample)</td>
<td>Replicates 5</td>
</tr>
<tr>
<td>Sample solution - I</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Sample solution - II</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>check standard</td>
<td>Replicate 2</td>
</tr>
</tbody>
</table>

An example of sequence for change Column oven temperature (35°C)

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>No. of Injections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluent Blank Sample</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Standard Sample (Reference Sample)</td>
<td>Replicates 5</td>
</tr>
<tr>
<td>Sample solution - I</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Sample solution - II</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>check standard</td>
<td>Replicate 2</td>
</tr>
</tbody>
</table>
**Change in pH -buffer:**
(Normal Experimental Condition: pH -buffer 6.00)

An example of sequence for change in pH -buffer (5.80)

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>No. of Injections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluent Blank Sample</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Standard Sample (Reference Sample)</td>
<td>Replicates 5</td>
</tr>
<tr>
<td>Sample solution - I</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Sample solution - II</td>
<td>Replicate 1</td>
</tr>
</tbody>
</table>

An example of sequence for change in pH -buffer (6.20)

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>No. of Injections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluent Blank Sample</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Standard Sample (Reference Sample)</td>
<td>Replicates 5</td>
</tr>
<tr>
<td>Sample solution - I</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Sample solution - II</td>
<td>Replicate 1</td>
</tr>
</tbody>
</table>
Change in Wavelength:
(Normal Experimental Condition: 225 Nanometer)

An example of sequence for change in Wavelength (223 Nanometer)

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>No. of Injections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluent Blank Sample</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Standard Sample (Reference Sample)</td>
<td>Replicates 5</td>
</tr>
<tr>
<td>Sample solution - I</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Sample solution - II</td>
<td>Replicate 1</td>
</tr>
</tbody>
</table>

An example of sequence for change in Wavelength (227 Nanometer)

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>No. of Injections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluent Blank Sample</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Standard Sample (Reference Sample)</td>
<td>Replicates 5</td>
</tr>
<tr>
<td>Sample solution - I</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Sample solution - II</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>check standard</td>
<td>Replicate 2</td>
</tr>
</tbody>
</table>

Data observation:
Suitability parameter for system is to be reported for each experiment.
% RSD between six results of Precision of Method and two results obtained with changed condition is to be reported for each experiment.
Stability of Analytical Sample:

Experiment:

Prepare standard Sample (Reference Sample), samples that are test Sample of Montelukast Sodium Chewable Tablets 5 mg at the beginning of this exercise i.e. on 1\textsuperscript{st} day and on 3\textsuperscript{rd}, 5\textsuperscript{th}, 6\textsuperscript{th}, 7\textsuperscript{th} day of experiment. Store the Sample at room temperature for every time interval up to 8\textsuperscript{th} day. Analyze these Samples on 8\textsuperscript{th} day with freshly prepared test Sample. Prepare standard Sample (Reference Sample) freshly at the time for analysis and calculate the % assay of Montelukast in freshly prepared and stored test Samples.

The sequence of injections for Sample stability is given as:

An example of sequence for Sample Stability

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>No. of Injections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluent Blank Sample</td>
<td>2</td>
</tr>
<tr>
<td>Freshly prepared standard Sample</td>
<td>5</td>
</tr>
<tr>
<td>(Reference Sample)</td>
<td></td>
</tr>
<tr>
<td>7\textsuperscript{th} day prepared standard</td>
<td>1</td>
</tr>
<tr>
<td>6\textsuperscript{th} day prepared standard</td>
<td>1</td>
</tr>
<tr>
<td>5\textsuperscript{th} day prepared standard</td>
<td>1</td>
</tr>
<tr>
<td>3\textsuperscript{rd} day prepared standard</td>
<td>1</td>
</tr>
<tr>
<td>1\textsuperscript{st} day prepared standard</td>
<td>1</td>
</tr>
<tr>
<td>Freshly prepared Test Sample – 8\textsuperscript{th} day</td>
<td>1</td>
</tr>
<tr>
<td>Freshly prepared Test Sample – 8\textsuperscript{th} day</td>
<td>1</td>
</tr>
<tr>
<td>Freshly prepared Test Sample – 8\textsuperscript{th} day</td>
<td>1</td>
</tr>
<tr>
<td>7\textsuperscript{th} day prepared Test Sample</td>
<td>1</td>
</tr>
<tr>
<td>6\textsuperscript{th} day prepared Test Sample</td>
<td>1</td>
</tr>
<tr>
<td>Bracketing standard_1</td>
<td>2</td>
</tr>
<tr>
<td>5\textsuperscript{th} day prepared Test Sample</td>
<td>1</td>
</tr>
<tr>
<td>3\textsuperscript{rd} day prepared Test Sample</td>
<td>1</td>
</tr>
<tr>
<td>1\textsuperscript{st} day prepared Test Sample</td>
<td>1</td>
</tr>
<tr>
<td>Sample Name</td>
<td>No. of Injections</td>
</tr>
<tr>
<td>--------------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>Bracketing standard_2</td>
<td>2</td>
</tr>
</tbody>
</table>

**Data evaluation:**

The test Sample for Montelukast Sodium Chewable Tablets 5 mg is said to be stable up to the time point till which the % RSD of the results of stored test Sample when compared to the result of freshly prepared test Sample is less than 2.0.

Calculate the % recovery of the stored standard Sample (Reference Sample) against freshly prepared standard Sample (Reference Sample).