Chapter: 2

Review - Literature
2.1. Literature on Different Method development and validation:

It is found that world grows in sector of pharmaceutical industries very fast. It is due to its need in daily routine it is essential to get medication. We see that the cost of medicines are too high so that people does not get medicines on proper time. So it is found that the death rate increases in day today’s life. The important question is that how to reduce the rate of medicine? So that people take medicine. If we want to reduce the cost of medicines, it required to reduce the manufacturing cost. The cost of any finished product is depending on its manufacturing cost. It is well known to all of us that pharmaceutical industries spend their money in the quality approach. Because quality of medicine is directly approach with human and animal health. Most of the testing of pharmaceutical product is carried out on different quality checking instruments. From all of these instruments about 60% analysis done on HPLC (Liquid chromatography with high pressure).

Now a days the Anti HIV and anticancer drugs are very important. The research work going on for these drugs very fastly as its need. The Most of private sector and Government research center are interested to work find out the complete Sample for this disease.

The HIV spread from year 2000. Currently India spend 5% of it health budget on health sector for this disease. The vast growing cancer also very critical issues to solve.

So in this project method for Anti HIV drugs is to be develop and validated as per norms and condition. The method trying to develop is very cheap, easy to analyze. the solvent used are very easily available.

Some Anti HIV Molecules from literature are Lamivudine, Zidovudine, Ritonavir, Lopinavir, Atazanavir, Emtricitabine, Tenofovir Disoproxil fumrate, Efavirenz.
Structure: Lamivudine

Structure: Zidovudine
Structure: Atazanavir Sulphate

Molecular wt-of Atazanavir Sulphate : 802.9
Structure: Ritonavir

Ritonavir mol-formula : $\text{C}_{37}\,\text{H}_{48}\,\text{N}_{6}\,\text{O}_{5}\,\text{S}_{2}$
Molecular wt-of Ritonavir : 720.95
2.1.1 A validated Impurity Determination Method for Montelukast by liquid chromatography.

System Controlling Parameter

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
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<tr>
<td>Name of system</td>
<td>HPLC</td>
</tr>
<tr>
<td>Column used</td>
<td>dC18, 250Mili miter x 4.6 Mili meter x 5 um Atlantis</td>
</tr>
<tr>
<td>Flow-rate</td>
<td>1.5 ml / minute</td>
</tr>
<tr>
<td>Absorption Wavelength</td>
<td>225 Nanometer</td>
</tr>
<tr>
<td>Temperatures of Column oven</td>
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</tr>
<tr>
<td>Volume at time of injection</td>
<td>20 µl</td>
</tr>
<tr>
<td>Mode type For Analysis</td>
<td>Gradient</td>
</tr>
</tbody>
</table>

A new developed validated reverse phase HPLC method proposed in this article. The most important thing is that, this method is stability indicating method because this method carried out forced degradation study successfully on different condition like hydrogen per oxide degradation, Acid-alkali degradation. This is an impurity determination method and the Montelukast sodium are completely resolved (Re-solution) from each other and principle peak in standard Sample (Reference Sample) of 2 mg/ml (2PPM) has within the limit.

This is gradient method in which Atlantis dC18, 250cm x 4.6 Mili meter x 5 um column was used having flow of 1.5 ml/min, column temperature 20°C, injected sample of 20 ul detected at 225 Nanometer wave length. But after reviewing the chromatogram of this method it is observed that impurity-D is very close after principle peak so it is required to resolved impurity - D from the principle peak.
2.1.2 A validated determination-method for Irbesartan tablets:

**System Controlling Parameter**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Specification</th>
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</thead>
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<tr>
<td>Name of system</td>
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</tr>
<tr>
<td>Column used</td>
<td>Inertisil ODS, C-18, 250cm x 4.6 Mili meter x 5 um</td>
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<tr>
<td>Flow-rate</td>
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<td>Absorption Wavelength</td>
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<tr>
<td>Temperatures of Column oven</td>
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<tr>
<td>Volume at time of injection</td>
<td>20 µl</td>
</tr>
<tr>
<td>Mode type For Analysis</td>
<td>Gradient</td>
</tr>
</tbody>
</table>

This research paper proposed an isocratic method for determination of Irbesartan in respective dosages form. An inertisil ODS, C-18, 250cm x4.6 mm x 5 um column used by applying 1.5 ml /min at wave length 260 Nanometer.

A mixture of ACN (Acetonitrile), Methanol, 2 % Phosphoric acid Sample (40:40:20 v/v/v) were use in preparation of phase. The Main peak observed at 4.5 min. Validation parameter also carried out as per ICH guideline. But after reviewing Chromatogram , it is possible to reduce the cost of analysis by using low proportion of solvent, high proportion of 2 % Phosphoric acid Sample and same make of column having 5 cm or 15 cm length instead of 25 cm , it may be achieve same retention with simple reverse phase method.
2.1.3 Determination of Azithromycin and ambroxol in tablets by liquid chromatography.

**System Controlling Parameter**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name of system</td>
<td>HPLC</td>
</tr>
<tr>
<td>Column used</td>
<td>phenomenex Gemini C-18 column of 25cm x 4.6 Mili meterx</td>
</tr>
<tr>
<td>Flow-rate</td>
<td>2.0 ml / minute</td>
</tr>
<tr>
<td>Absorption Wavelength</td>
<td>220 Nanometer</td>
</tr>
<tr>
<td>Temperature of Column oven</td>
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<tr>
<td>Volume at time of injection</td>
<td>10 µl</td>
</tr>
<tr>
<td>Mode type For Analysis</td>
<td>Isocratic.</td>
</tr>
</tbody>
</table>

In this the combination of two drugs are estimated under a single reverse phase chromatographic method. The separation of both peaks was carried out by using Potassium di hydrogen phosphate in water (pH 8.5) and ACN (Acetonitrile) respectively 350 ml and 650 ml.

The C-18 Column of Gemini (Phenomenex) of 25cm x 4.6 Mili meterx 5u, used. The column having flow of per minute 2.0 ml and 220 Nanometer wavelengths. The linearity over concentration range of 96-145 ug/ml and 80-125 ug/ml assay of respective component. Amroxol and Azithromysin were found at Elution time for 3.7 min and 6.1 min respectively. Validation also carried out for this method successfully so this method is very simple for estimation of the respective drug at same time.
2.1.4 Rabeprazole Sodium-ultra performance Liquid-chromatography to determine sample:

**System Controlling Parameter**

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<th>Specification</th>
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<td>UPLC</td>
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<tr>
<td>Column used</td>
<td>C-18, (50- millimeter x 2.1 millimeter x 1.7 um Aquity BEH)</td>
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<tr>
<td>Flow-rate</td>
<td>0.4 milli liter / minute</td>
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<td>Absorption Wavelength</td>
<td>280 - Nanometer</td>
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<td>Temperatures of Column oven</td>
<td>Ambient Temp.</td>
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<td>5.0 µl</td>
</tr>
<tr>
<td>Mode type For Analysis</td>
<td>Isocratic.</td>
</tr>
</tbody>
</table>

This is an advanced technique of HPLC and required very less time to analysis. In this method the separation achievement through stationary phase column water Aquity C-18, (50 millimeter x 2.1 millimeter x 1.7 um BEH column. The mixture of pH 7.4 buffer of potassium Dihydrogen phosphate and ACN (Acetonitrile) used in ratio 65:35 v/v.

The 0.4 ml /min flow pumped through system, 5.0 ul and 280 Nanometer having run time for 2.0 min. It is found that % RSD was within 1.5 %. This method is very used full for Sample .In extended release dosages forms it is required to withdrawal samples at 24 hours time interval. Some time there are more time points so it required 2 days to obtained results in HPLC method. But instead of HPLC if we used UPLC it required only 2-3 hours to complete Analysis.

So after reviewing of most of literature it is found that HPLC required different types of column, chemicals, and impurities. And all these are very costly in market. If analytical person develop simple, short run time and less chemical consumable analytical method of analysis. It is possible to reduce the cost of medicine. so, as the cost of manufacturing reduce, the cost of finish product also reduce so that people are easily purches the medicine, and automatically it is found that the growth rate of death is also reduces.
2.1.5 Validated method after development for montelukast ::-
(Patel SV, Patel GF, Pipaliya SG, Pharma Ana & Qual Assur Vol 2012, Issue 2)
This Estimated method used to determination of Montelukast. This instrumental method acheived by first order derivatives and it defined at wave length of 339 Nanometer this instrumental method developed to estimate active content from combinaded drugs. The validated instrumental method beneficial but it was not carried out on high sensitive instrument like HPLC.

2.1.6 Determination of Montelukast Na and Fexofenadine Hcl in combined dosages form by HP-TLC
A HPTLC-method was determine for Montelukast Sodium from combined dosages form separation was carried out on hptlc aluminum plate (Merck) G60, F254, (208 x10 cm) with 250 um thickness. The Mobile phase selected for this instrumental method ethyl acetate: Methanol: ammonia (30 %) in proportion of 70:30:0.50 under this all the method does not show the impurity concern with Montelukast.

2.1.7 HPTLC method for validation of antiastamatic drugs: Method used for stability study during self life of drug.
(Patel Nilam, Patel shirish, Patel dhara, IJPRS, ISSN NO: 2277-7873V-1I-2 2012)
The main purpose of this method is used for stability study during self life of drug or respective product Monelukast sodium and other antiastamatic drugs impurity generated during forced degradation are resoved from each other and any other peak. The drug treated to acid, base, peroxide and photo degradation product form on aluminium backed silica gel 60 F254, plate’s instrumental method used to estimate different drugs.
2.1.8 Analytical Method with for stability study for Montelukast sodium and its photo degradation study in pharma dosages form.

(Journal chromatogr sci.2011:49 (7):540-6 21801485)

This instrumental method developed by Juliana R, Brier Ana R, Juliana R, for assay determination of Montelukast sodium in coated tablets. In this method Zorbax XDB C18 column was used. The mobile phase was selected water: ACN (Acetonitrile) : methanol in proportion of 150 ml:750ml : 100 ml and its pH 3.8 at 0.8 ml per minute of flow rate, the wave length selected for this instrumental method 280 Nanometer and its showed detector response for Montelukast sodium over the concentration range from 5-35 ul.in this method specificity also proved at stress condition. Montelukast Literature show much method for determination of Montelukast but no gradient instrumental method found as all impurities was well separated from each other so it is need to developed gradient pattern for accurately determination of montelukast from its dosages as tablets are not official in United state pharmacopeia.

2.1.9 Estimation of eszopiclone by using different analytical method Dosages Forms:


This instrumental method is developing by validating rapid assay of eszopiclone in bulk samples Tablets dosages forms. The method developed was isocratic method. The instrumental method developed as per standard analytical procedure. Different chromatographic condition was selected for estimation of Eszopiclone from tablets and bulk drug. Following chromatographic Conditon Selected for HPLC.
**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>C18, 15 centimeter x 4.6 millimeter, 5-µm, (Phenomenax Luna)</td>
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<tr>
<td>System flow-rate</td>
<td>1.0 milli liter / minute</td>
</tr>
<tr>
<td>Absorption Wavelength</td>
<td>304 Nanometer</td>
</tr>
<tr>
<td>Temperatures of Column oven</td>
<td>Ambient Temp.</td>
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<tr>
<td>Volume at time of injection</td>
<td>20-µl</td>
</tr>
<tr>
<td>Mode type For Analysis</td>
<td>Isocratic</td>
</tr>
</tbody>
</table>

From All Above literature study it was observed that it was need to established effective determination-method for both drugs. As instrumental method not official in United State Pharmacopeia and it was also challenge to separate all impurities from respective active drug.
3.1. Developed Analytical Method for Montelukast:

Method development for Dissolution of Montelukast in Montelukast Sodium chewable Tablets:

Dissolution Parameters:

- **Medium**: 0.5% SLS in Water.
- **Apparatus**: USP Type II [Paddle]
- **Volume**: 900 mL
- **RPM**: 50
- **Withdrawal Time**: 30 minutes
- **Temperature**: 37 ± 0.5°C

**Note**: Use amber color Dissolution jars for carrying out dissolution.

**Preparation of 0.5% SLS in Water**:

Dissolved 51.25 gram of SLS (lauryl sulphate) in 10 liters of water with constant stirring. Mixed well.

**Test Preparation**:

- Used six tablets at time of dissolution.
- Added 900 milliliter of dissolution medium to six vessels.
- Maintained temperature at 37 ± 0.5°C.
- Run instrument at specific time point
- Filter through filter to get supernant solution.

**System Controlling Parameter**

- **Name of system**: HPLC
- **Used Column**: C18 ,15 centi m x4.6 mili-meter5 µm (Waters Symmetry)
- **System flow-rate**: 1.5 milliliter / minute
- **Absorption Wavelength**: 240 Nano-meter
- **Temperatures of Column oven**: 40°C
- **Volume at time of injection**: 20 µL
- **Run time of injection**: 7 Minutes
- **Mode type For Analysis**: Isocratic system
**Preparation for mobile phase buffer solution:**
Take 2.725 gram of potassium phosphate (Dihydrogen) in 1 liter purified water, dissolved through wave sonicator. Make pH of this solution to 4.04 with help of diluted phosphoric acid. Filtered through 0.2 um filter paper and degassed.

**Preparation method for mobile phase:**
Take 400 milliliter of buffer solution and 1600 milliliter of acetonitrile in beaker. Mixed homoginiously and sonicate to degas.

**Diluent Blank Preparation method for diluents blank solution**
Used media as diluents blank solution for injection.

**Note:** Avoid Exposure of samples to light; Use low actinic Amber colored glass wares.

**Standard solution preparation for Montelukast:**
Take 31.12 milligram of Montelukast Na standard, into a 100 milliliter flask. Added 70 milliliter solvent methanol. Dissolved through wave sonicator. Diluted to volume with methanol homoginiously mixed and diluted 5 milliliter of this stock solution to 250 milliliter of dissolution media.

The effect of Sodium Vapour Lamp is studied on Dissolution experiment using transparent Dissolution Jars and Glassware’s along with amber color Dissolution Jars and Glassware’s.

**Buffer Preparation:**
Dissolved 2.702 gm of Potassium Orthophosphate (Dihydrogen) to 1 liter of purified water. Adjusted pH for this buffer solution to 4.00 ± 0.05 Unit with (O-phosphoric acid solution Filtered through 0.4 um filter.

**Dissolution Parameters:**
- Medium: 0.5% SLS in Water.
- Apparatus: USP Type II [Paddle]
- Volume: 900 mL
- RPM: 50
- Withdrawal Time: 30 minutes
- Temperature: 37 ± 0.5°C
**Preparation of 0.5% SLS in Water:**
Dissolved 50.124 gram of Lauryl Sulphate (SLS Make: Qualigens) in 10 liters of water with constant stirring. Mixed well.

**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>Used Column</td>
<td>C18, 15 centimeter x 4.6 millimeter 5 µm (Waters Symmetry)</td>
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<tr>
<td>System flow-rate</td>
<td>1.5 milliliter / minute</td>
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<tr>
<td>Absorption Wavelength</td>
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<tr>
<td>Temperatures of Column oven</td>
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<tr>
<td>Volume at time of injection</td>
<td>20 µL</td>
</tr>
<tr>
<td>Run time of injection</td>
<td>7 Minutes</td>
</tr>
<tr>
<td>Mode type For Analysis</td>
<td>Isocratic system</td>
</tr>
</tbody>
</table>
3.1.2 Method development of Content Uniformity of Montelukast sodium chewable Tablets: Chromatograms

Analytical method with spiking known impurities specified for drug substance.

**System Controlling Parameter**

- **Name of system**: HPLC
- **Used Column**: C18, 150 millimeter x 4.6 millimeter x 5 micron, Zorbax SB
- **System flow-rate**: 1.0 milliliter / minute
- **Absorption Wavelength**: 220 Nano-meter
- **Temperatures of Column oven**: 35°C
- **Sample Temperatures**: 10°C
- **Volume at time of injection**: 20 µL
- **Run time of injection**: 40 Minutes
- **Mode type For Analysis**: Gradient system
- **Used Diluent**: 75% Acetonitrile :25% Water.

**Gradient:**

<table>
<thead>
<tr>
<th>Time (Minutes)</th>
<th>Milliliter of flow per min.</th>
<th>Phase-A %</th>
<th>Phase-B %</th>
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<tr>
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<td>1.2</td>
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<td>40</td>
</tr>
<tr>
<td>3</td>
<td>1.2</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>7</td>
<td>1.2</td>
<td>39</td>
<td>61</td>
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<td>20</td>
<td>1.2</td>
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<td>1.2</td>
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<td>40</td>
</tr>
<tr>
<td>40</td>
<td>1.2</td>
<td>60</td>
<td>40</td>
</tr>
</tbody>
</table>
Preparation of Buffer:
Transferred 1.354 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: Used buffer

Mobile Phase B: Use 100% Acetonitrile as such of Merck
Literature (Monograph) Methods for active pharmaceutical ingredient and finish product.

Related substances determination of Efaverenz tablets:
System Controlling Parameter

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Used Column</td>
<td>25 cm x 4.6 millimeter 5 μm (Zorbax SB CN)</td>
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<td>System flow-rate</td>
<td>1.5 milliliter / minute</td>
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<tr>
<td>Absorption Wavelength</td>
<td>252 Nano-meter</td>
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<tr>
<td>Temperatures of Column oven</td>
<td>40°C</td>
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<tr>
<td>Temperatures for sampler</td>
<td>5°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>
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</table>

Gradient Programme:

<table>
<thead>
<tr>
<th>Time (Minutes)</th>
<th>Milliliter of flow per min.</th>
<th>Phase-A %</th>
<th>Phase-B %</th>
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</thead>
<tbody>
<tr>
<td>0</td>
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<td>43</td>
</tr>
<tr>
<td>20</td>
<td>1.5</td>
<td>57</td>
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<td>43</td>
</tr>
<tr>
<td>40</td>
<td>1.5</td>
<td>57</td>
<td>43</td>
</tr>
</tbody>
</table>

Preparation of Buffer:
Transferred 2.7321 gram Potassium Phosphate (monohydrate) in 1000 milliliter of purified water. Added 1 milliliter of TEA (Triethylamine) made the pH of buffer to 2.51 with dilute OPA(Orthophosphoric acid) degassed it by filtered through 0.45μ filter.
**Mobile Phase-A:** A mixture of buffer methanol (90:10)

**Mobile Phase B:** A mixture of buffer methanol (10:90)

**Diluent:** Water: Methanol (20:80).

**Efavirenz Standard preparation:**
Weighed 30.00 mg of Efavirenz 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. Further dilute 1ml to 100 ml.

**Making of Placebo Sample:**
Weighed and transferred 120 mg of placebo in 200 milli liter of flask to it add 100 milliliter of diluent dissolved through wave Sonication for 20 Min, diluted to volume (ring mark) of flask with help of diluents solution and filter.

**Test Sample Preparation:**
Crushed 20 Tablets, Transferred weighed content of tablets equal to 120 milli gram of efavirenz in 200 milli liter of flask. Added 100 ml of diluent, Sonicate it through occasional Slight Shaking for 20 Minutes in ice cold water dilute to neck mark of flask and filter it.
Related substances determination of Lopinavir in EMLETRA tablets:

**System Controlling Parameter**

Name of system                           : HPLC  
Used Column                               : 25 cm x 4.6 milli-meter 5 µm (Inersil ODS 3V)  
System flow- rate                         : 1.5 milliliter / minute  
Absorption Wavelength                    : 210 Nano-meter  
Temperatures of Column oven              : 25°C  
Volume at time of injection              : 10 µL  
Run time of injection                    : 55 Minutes  
Mode                                      : Isocratic

**Preparation of Buffer:**

Transferred 1.364 gram Potassium Phosphate (monohydrate) in 1000 milliliter of purified water, made the pH of buffer to 4.0 with dilute OPA (Orthophosphoric acid) degassed it by filtered through 0.45µ filter.

**Mobile Phase:** A mixture of buffer acetonitrile (55:45) 
**Diluent:** Mobile phase. 

**Lopinavir Standard preparation:**

Weighed 50.00 mg of Lopinavir 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. Further dilute 1ml to 100 ml.

**Making of Placebo Sample:**

Weighed and transferred 535.0 mg (Contains Ritonavir) of placebo in 100 milli liter of flask to it add 100 milliliter of diluent dissolved through wave Sonication for 15 Min, diluted to volume (ring mark) of flask with help of diluents solution and filter.

**Test Sample Preparation:** -

Crushed 20 Tablets, Transferred weighed content of tablets equal to 100 milli gram of Lopinavir in 100 milli liter of flask. Added 70 ml of diluent, Sonicate it through occasional Slight Shaking for 20 Minutes in ice cold water dilute to neck mark of flask and filter it.
Assay determination of Lopinavir and Ritonavir in EMLETRA tablets:

System Controlling Parameter

Name of system : HPLC
Used Column : 15 cm x 4.6 millimeter 5 µm (Hypersil BDS)
System flow-rate : 1.5 milliliter / minute
Absorption Wavelength : 210 Nano-meter
Temperatures of Column oven : 25°C
Volume at time of injection : 20 µL
Run time of injection : 10 Minutes
Mode : Isocratic

Preparation of Buffer:
Transferred 6.800 gram Potassium Phosphate (monohydrate) in 1000 milliliter of purified water, made the pH of buffer to 3.0 with dilute OPA (Orthophosphoric acid) degassed it by filtered through 0.45µ filter.

Solvent Mixture: Acetonitrile: methanol (80:200)

Mobile Phase: A mixture of buffer and solvent (45:55)

Diluent : Methanol /Mobile phase.

Standard preparation:
Weighed 80.00 mg of Lopinavir and 20.00 mg Ritonavir working standard to 100.0 milliliter of flask to it added 75 milliliter diluents solution dissolved it through wavenicater and diluted this flask to its mark level through help of diluent solution. Further dilute 5 ml to 50 ml.

Test Sample Preparation: -
Crushed 20 Tablets, Transferred weighed content of tablets equal to 200 milli gram of Lopinavir in 250 milli liter of flask. Added 170 ml of methanol Sonicate it through occasional Slight Shaking for 25 Minutes dilute to neck mark of flask and filter it. Further dilute 5 ml to 50 ml.
Related substances determination of Ritonavir in EMLETRA tablets:

System Controlling Parameter
Name of system : HPLC
Used Column : 15 cm x 4.6 mili-meter, 3 µm (YMC C4)
System flow-rate : 1.0 milliliter / minute
Absorption Wavelength : 210 Nano-meter
Temperatures of Column oven : 60°C
Volume at time of injection : 50 µL
Run time of injection : 135 Minutes
Mode : Gradient

Gradient Programme:

<table>
<thead>
<tr>
<th>Time (Minutes)</th>
<th>Milliliter of flow per min.</th>
<th>Phase-A %</th>
<th>Phase-B %</th>
</tr>
</thead>
<tbody>
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<td>0</td>
<td>1.0</td>
<td>100</td>
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</tr>
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<td>1.0</td>
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</tr>
<tr>
<td>135</td>
<td>1.0</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

Preparation of Buffer:
Transferred 4.100 gram Potassium Phosphate (monohydrate) in 1000 milliliter of purified water degassed it by filtered through 0.45µ filter.

Mobile Phase A : A mixture of buffer, acetonitrile, tetrahydrofuran, butanol (690:180:80:50)
Mobile Phase B : A mixture of buffer, acetonitrile, tetrahydrofuran, butanol (400:470:80:50)

Diluent : Buffer : CAN (30:70)

Ritonavir Standard preparation:
Weighed 50.00 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. Further dilute 1ml to 100 ml.
**Making of Placebo Sample:**
Weighed and transferred 535.0 mg (Contains Lopinavir) of placebo in 100 milli liter of flask to it add 100 milliliter of diluent dissolved through wave Sonication for 15 Min, diluted to volume (ring mark) of flask with help of diluents solution and filter.

**Test Sample Preparation:**
Crushed 20 Tablets, Transferred weighed content of tablets equal to 100 milli gram of Ritonavir in 100 milli liter of flask. Added 70 ml of diluent Sonicate it through occasional Slight Shaking for 20 Minutes in ice cold water dilute to neck mark of flask and filter it.
Assay analytical method for determination of Efavirenz tablets:

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>Used Column</td>
<td>C 18, 75 mm x 4.6 millimeter 3.5 μm (Water symmetry)</td>
</tr>
<tr>
<td>System flow-rate</td>
<td>1.5 milliliter / minute</td>
</tr>
<tr>
<td>Absorption Wavelength</td>
<td>252 Nano-meter</td>
</tr>
<tr>
<td>Temperatures of Column oven</td>
<td>45°C</td>
</tr>
<tr>
<td>Volume at time of injection</td>
<td>20 μL</td>
</tr>
<tr>
<td>Run time of injection</td>
<td>15 Minutes</td>
</tr>
</tbody>
</table>

Preparation of Buffer:
Transferred 8.600 gram Ammonium Dihydrogen orthophosphate in 1000 milliliter of purified water, made the pH of buffer to 3.00 with dilute OPA (Orthophosphoric acid) degassed it by filtered through 0.45μ filter.

Mobile Phase: A mixture of buffer and acetonitrile (58:42)

Diluent: Methanol (100%)

Efavirenz Standard preparation:
Weighed 30.00 mg of Efavirenz 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. Further dilute 10 ml to 50 ml with mobile phase.

Test Sample Preparation:
Crushed 20 Tablets, Transferred weighed tablet powder about 240 mg to 200 milliliter of flask. Added 100 ml of diluent Sonicated it through occasional Slight Shaking for 20 Minutes dilute to neck mark of flask and filter it. Further dilute 10 ml to 50 ml with mobile phase.
Chromatographic purity method for Lamivudine USP: (monographic method)

System Controlling Parameter

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name of system</td>
<td>HPLC</td>
</tr>
<tr>
<td>Used Column</td>
<td>25 cm x 4.6 milli-meter, 5µm (Inertsil ODS 2)</td>
</tr>
<tr>
<td>System flow-rate</td>
<td>1.0 milliliter / minute</td>
</tr>
<tr>
<td>Absorption Wavelength</td>
<td>277 Nano-meter</td>
</tr>
<tr>
<td>Temperatures of Column oven</td>
<td>35°C</td>
</tr>
<tr>
<td>Volume at time of injection</td>
<td>10 µL</td>
</tr>
<tr>
<td>Run time of injection</td>
<td>45 Minutes</td>
</tr>
<tr>
<td>Mode</td>
<td>Isocratic</td>
</tr>
</tbody>
</table>

Preparation of Buffer:
Transferred 1.90 gram Ammonium acetate in 1000 milliliter flask added 90 ml purified water, made the pH of buffer to 3.8 with dilute acetic acid dilute to 1000 milliliter with help of water degassed it by filtered through 0.45µ filter.

Mobile Phase: A mixture of buffer, Methanol (95:5)

Diluent: Mobile phase

Making of Standard preparation:
Weighed 5.00 mg of salicylic acid and 5.00 mg Lamivudine working standard to 100.0 milliliter of flask to it added 75 milliliter diluents solution dissolved it through wave sonicater and diluted this flask to its mark level through help of diluent solution. Further dilute 1ml to 25 ml with help of mobile phase.

Test Sample Preparation: -
Transferred weighed 20.00 milligram test sample in 10 milli liter of flask. Added 7 ml of diluent Sonicate it through occasional Slight Shaking for 3 Minutes dilute to neck mark of flask and filter it.
Method for Dissolution of Ritonavir in Ritonavir tablets IP 100 mg:

**Dissolution Parameter**

- **Medium**: 0.1 M Hydrochloric acid
- **Apparatus**: IP Type I (paddle)
- **Volume**: 900 milliliter
- **RPM**: 100
- **Withdrawal time**: 60 Minutes
- **Temperature**: 37 ± 0.5°C

**Test Preparation:**
Carried out test for dissolution on six tablets. Placed one tablets on each vessels containing 900 ml of medium attending demonstrated temp ie 37 °C. Run instrument and withdrawal sample after specified method time. Filter sample through filter paper.

**System Controlling Parameter**

- **Name of system**: HPLC
- **Used Column**: 5 cm x 4.6 millimeter 3 µm (Phenyl)
- **System flow-rate**: 2.5 milliliter / minute
- **Absorption Wavelength**: 239 Nano-meter
- **Temperatures of Column oven**: 45°C
- **Volume at time of injection**: 10 µL
- **Run time of injection**: 7 Minutes
- **Mode**: Isocratic

**Preparation of Buffer:**
Transferred 3.400 gram sodium acetate trihydrate and 0.94 gram of sodium 1-hexane sulphonate in 1000 milliliter of purified water, made the pH of buffer to 4.0 with dilute hydrochloric acid degassed it by filtered through 0.45µ filter.

**Mobile Phase:** A mixture of buffer and Acetonitrile (55:45)

**Diluent**: Methanol / Dissolution media.
**Standard preparation:**
Weighed 100.0 mg of Ritonavir working standard to 100.0 milliliter of flask to it added 75 milliliter Methanol solution dissolved it through wave sonicater and diluted this flask to its mark level through help of Methanol solution. Further dilute 5 ml to 50 ml with help of dissolution media.
Assay determination of Ritonavir in Ritonavir Tablets IP:

System Controlling Parameter

Name of system : HPLC
Used Column : 5 cm x 4.6 mili-meter, 3µm (Phenyl)
System flow-rate : 2.5 milliliter / minute
Absorption Wavelength : 239 Nano-meter
Temperatures of Column oven : 45°C
Volume at time of injection : 10 µL
Run time of injection : 7 Minutes
Mode : Isocratic

Preparation of Buffer:
Transferred 3.400 gram sodium acetate trihydrate and 0.94 gram of sodium 1-hexane sulphonate in 1000 milliliter of purified water, made the pH of buffer to 4.0 with dilute hydrochloric acid degassed it by filtered through 0.45µ filter.

Mobile Phase: A mixture of buffer and solvent (55:45)

Diluent: Methanol.

Standard preparation:
Weighed 25.00 mg of Ritonavir working standard to 100.0 milliliter of flask to it added 75 milliliter diluents solution dissolved it through wave sonicater and diluted this flask to its mark level through help of diluent solution.

Test Sample Preparation: -
Crushed 20 Tablets, Transferred weighed content of tablets equal to 25.0 milli gram of Ritonavir in 100 milli liter of flask. Added 70 ml of methanol sonicate it through occasional Slight Shaking for 20 Minutes dilute to neck mark of flask and filter it.
Analytical method for Limit of Enantiomer of Lamivudine USP:
(Monographic Method)

System Controlling Parameter

- **Name of system**: HPLC
- **Used Column**: 250 mm x 4.6 millimeter, 4.6 μm (Chiral pak)
- **System flow-rate**: 0.5 milliliter / minute
- **Absorption Wavelength**: 270 Nano-meter
- **Temperatures of Column oven**: 25°C
- **Volume at time of injection**: 10 μL
- **Run time of injection**: 20 Minutes
- **Mode**: Isocratic

Mobile Phase:
Mixed 900 milliliter of ethanol, 100 milliliter of isopropyl alcohol and 1 milliliter of diethyl amine filter it

**Diluent**: Mobile phase

**Standard preparation:**
Weighed 2.5 mg of Lamivudine Resolution mixture –A RS to 10.0 milliliter of flask to it added 7 milliliter diluents solution dissolved it through wave sonicater and diluted this flask to its mark level through help of diluent solution.

**Test Sample Preparation:** -
Transferred weighed 2.5 milli gram of test sample in 10 milli liter of flask. Added 7 ml of diluent sonicate it through occasional Slight Shaking for 2 Minutes dilute to volume and filter it.
**Assay- Analytical method for Lamivudine, Zidovudine and Nevirapine from its Tablets Form:**

**System Controlling Parameter**

- **Name of system**: HPLC
- **Used Column**: 250 mm x 4.6 mili-meter, 5 µm (Cyno)
- **System flow- rate**: 1.0 milliliter / minute
- **Absorption Wavelength**: 270 Nano-meter
- **Temperatures of Column oven**: Ambient
- **Volume at time of injection**: 20 µL
- **Run time of injection**: 40 Minutes
- **Mode**: Isocratic

**Mobile Phase:**
Mixed 150 milliliter of Acetonitrile to 850 milliliter of water filter and degassed.

**Diluent**: Methanol, water (50:50)

**Lamivudine Standard preparation: (Stock-A)**
Weighed about 15.0 milligram of Lamivudine standard to 100.0 milliliter of flask to it added 75 milliliter diluents solution dissolved it through wave sonicater and diluted this flask to its mark level through help of diluent solution.

**Zidovudine Standard preparation: (Stock-B)**
Weighed about 30.0 milligram of Zidovudine standard to 100.0 milliliter of flask to it added 75 milliliter diluents solution dissolved it through wave sonicater and diluted this flask to its mark level through help of diluent solution.

**Nevirapine Standard preparation: (Stock-C)**
Weighed about 30.0 milligram of Nevirapine standard to 100.0 milliliter of flask to it added 75 milliliter diluents solution dissolved it through wave sonicater and diluted this flask to its mark level through help of diluent solution.
Mixed Standard preparation:
Transferred 10.0 ml of each stock A, B, C, in 100 milli liter of flask and dilute to volume with help of diluent mixed.

Test Sample Preparation: -
Crushed 20 Tablets, Transferred weighed content of tablets equal to 15 milli gram of Lamivudine , 30 milli gram of Zidovudine , 30 milli gram of Nevirapine , in 100 milli liter of flask. Added 70 ml of diluent, Sonicate it through occasional Slight Shaking for 20 Minutes in ice cold water dilute to neck mark of flask and filter it.
Dissolution - Analytical method for Lamivudine, Zidovudine and Nevirapine from its Tablets Form:

Dissolution Parameter

Medium : 0.1 M Hydrochloric acid
Apparatus : USP Type II (paddle)
Volume : 900 milliliter
RPM : 50
Withdrawal time : 5, 10, 15, 20, 30, 45 Minutes
Temperature : 37 ± 0.5°C

Test Preparation:
Carried out test for dissolution on six tablets. Placed one tablets on each vessels containing 500 ml of medium attending demonstrated temp ie 37 °C. Run instrument and withdrawal sample after specified method time. Filter sample through filter paper.

System Controlling Parameter

Name of system : HPLC
Used Column : 250 mm x 4.6 mili-meter, 5 µm (Cyno)
System flow-rate : 1.0 milliliter / minute
Absorption Wavelength : 270 Nano-meter
Temperatures of Column oven : Ambient
Volume at time of injection : 25 µL
Run time of injection : 10 Minutes
Mode : Isocratic

Mobile Phase:
Mixed 200 milliliter of Acetonitrile to 800 milliliter of water filter and degassed.

Diluent: Medium.
**Lamivudine Standard preparation: (Stock-A)**
Weighed about 20.0 milligram of Lamivudine standard to 100.0 milliliter of flask to it added 75 milliliter diluent solution dissolved it through wave sonicater and diluted this flask to its mark level through help of diluent solution.

**Zidovudine Standard preparation: (Stock-B)**
Weighed about 40.0 milligram of Zidovudine standard to 100.0 milliliter of flask to it added 75 milliliter diluent solution dissolved it through wave sonicater and diluted this flask to its mark level through help of diluent solution.

**Nevirapine Standard preparation: (Stock-C)**
Weighed about 35.0 milligram of Nevirapine standard to 100.0 milliliter of flask to it added 75 milliliter diluent solution dissolved it through wave sonicater and diluted this flask to its mark level through help of diluent solution.

**Mixed Standard preparation:**
Transferred 2.0 ml of each stock A, B, C, in 50 milli liter of flask and dilute to volume with help of diluent mixed.
Related substances - Analytical method for Lamivudine, Zidovudine and Nevirapine from its Tablets Form:

System Controlling Parameter

Name of system : HPLC
Used Column : 250 mm x 4.6 milli-meter, 5 µm (Inertsil)
System flow-rate : 1.0 milliliter / minute
Absorption Wavelength : 270 Nano-meter
Temperatures of Column oven : 35°C
Volume at time of injection : 25 µL
Run time of injection : 60 Minutes
Mode : Isocratic

Gradient Programme:

<table>
<thead>
<tr>
<th>Time (Minutes)</th>
<th>Milliliter of flow per min.</th>
<th>Phase-A %</th>
<th>Phase-B %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>1.0</td>
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</tr>
<tr>
<td>25</td>
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</tr>
<tr>
<td>60</td>
<td>1.0</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

Preparation of Buffer:
Transferred 6.80 gram Potassium Phosphate (monohydrate) in 1000 milliliter of purified water.

Mobile Phase A:
Mixed 130 milliliter of methanol to 870 milliliter of buffer made up it pH to 6.5 with Triethylamine, filter and degassed.

Mobile Phase B:
Mixed 400 milliliter of Acetonitrile to 600 milliliter of buffer made up it pH to 6.5 with Triethylamine, filter and degassed.

**Diluent:** Methanol: water (50:50)

**Lamivudine Standard preparation:** *(Stock-A)*

Weighed about 15.0 milligram of Lamivudine standard to 100.0 milliliter of flask to it added 70 milliliter diluent solution dissolved it through wave sonicater and diluted this flask to its mark level through help of diluent solution.

**Zidovudine Standard preparation:** *(Stock-B)*

Weighed about 30.0 milligram of Zidovudine standard to 100.0 milliliter of flask to it added 70 milliliter diluent solution dissolved it through wave sonicater and diluted this flask to its mark level through help of diluent solution.

**Nevirapine Standard preparation:** *(Stock-C)*

Weighed about 30.0 milligram of Nevirapine standard to 100.0 milliliter of flask to it added 70 milliliter diluent solution dissolved it through wave sonicater and diluted this flask to its mark level through help of diluent solution.

**Mixed Standard preparation:**

Transferred 1.0 ml of each stock A, B, C, in 100 milli liter of flask and dilute to volume with help of diluent mixed.

**Test Sample Preparation:** -

Crushed 20 Tablets, Transferred weighed content of tablets equal to 30 milli gram of Lamivudine , 60 milli gram of Zidovudine , 60 milli gram of Nevirapine , in 200 milli liter of flask. Added 150 ml of diluent, Sonicate it through occasional Slight Shaking for 20 Minutes in cold water dilute to neck mark of flask and filter it.