3.0. Analytical and bioanalytical method development

The term “Chromatography” is applied to wide variety of separation techniques which utilizes sample partitioning between the mobile phase and stationary phase as the basis for separation. Analyte gets distributed and equilibrated between the two phases during passage of mobile phase containing analyte through the stationary phase. Analyte gets retained on the stationary phase in proportion to its affinity towards stationary phase. Chromatographic system operating at equilibrium indicates that, at any given time, analyte may be in the mobile phase or in the stationary phase or not moving at all. The physicochemical property of the analyte controls partitioning/differential migration between the two phases which provides the basis for separation (Stout and Dorsey, 2002).

High Performance Liquid Chromatography (HPLC) is widely used analytical technique for separation and estimation of the analyte which can be applied in all stages of drug discovery, development (pre-clinical and clinical studies) and production (Kazakevich and Lobrutto, 2007). Physicochemical properties of analyte like chemical structure, molecular weight, pKa values, UV spectra of the compound, solubility in various buffers, solvents and concentration range of analyte that is present in the sample of interest should be known before starting method development (Snyder et al., 1997).

Selective, sensitive and simple analytical method for estimation of drug in biological fluids (i.e. serum, plasma, urine, cerebrospinal fluid) is essential for the successful conduct of preclinical and biopharmaceutical studies. Formulation studies which are designed to improve solubility and dissolution demand in vivo pre-clinical bioavailability studies to prove the hypothesis. Such pre-clinical bioavailability investigations require the support of a fast and reliable bioanalytical method for the measurement of the drug involved. Bioanalytical method development involves collection of blood, separation of plasma from the blood, sample preparation and extraction of analyte from biological matrix. Sample preparation is an important step during method development to ensure 100% of analyte in a solution for analysis. Different procedures such as protein precipitation, liquid-liquid extraction and solid phase extraction are routinely adopted to extract the drug from the biological matrix.
a. Protein precipitation method

This method is popular as more number of samples can be processed in a short time and is easy to use. Water miscible organic solvents (like acetonitrile, methanol, mixture of acetonitrile and methanol), and acids (like perchloric acid, trifluoroacetic acid and trichloroacetic acid) can be used as protein precipitating reagents. Generally three times higher protein precipitating reagent is added to the plasma sample containing analyte and an internal standard. The sample is mixed and centrifuged to separate the supernatant from the protein pellet. Supernatant can be directly injected into the column or dried and reconstituted before injecting. However this method suffers from various drawbacks such as method can be applied to small molecules and lacks specificity and selectivity. (Biddlecombe and Smith, 2004).

b. Liquid-liquid extraction method

This method involves mass transfer of the compound i.e. analyte present in the aqueous sample (plasma) is transferred into organic solvent having preferential selectivity for the analyte. Transfer of analyte from plasma into organic solvent depends on the selected organic solvent, polarity of the solvent and solvent mixture, pH of the aqueous phase and ratio of volume of the organic phase to aqueous phase. However, extraction of highly polar compounds is difficult with this method. Formation of emulsion is another problem during extraction process which may cause loss of analyte leading to low recovery (Biddlecombe and Smith, 2004).

c. Solid phase extraction (SPE)

Solid sorbent material with alkyl bonded silica packed in the cartridge is used for extraction of analyte from the plasma. Nature of the hydrocarbon bonded phase to the silica, solvent strength and chemical characteristics of the analyte are to be considered before optimization of extraction procedure. Sample recovery depends on the following factors

- Conditioning the sample for retention on the bed
- Equilibrate SPE bed with water
- Load sample onto the SPE bed
- Eluting retained analyte with a suitable organic solvent (Bakhtiar et al., 2007).
3.1. Analytical and bioanalytical method development for candesartan cilexetil

3.1.1. Identification of drug substance

a) **UV spectroscopy**
Primary stock solution of 1.0 mg/mL candesartan cilexetil was prepared by dissolving 10.0 mg of the drug in 10.0 mL of methanol. From this, 10.0 µg/mL of solution was prepared by serial dilutions. This 10.0 µg/mL solution was scanned between the wavelengths of 400 - 200 nm (UV spectrophotometer, UV-1601, Shimadzu, Kyoto, Japan) to determine the maximum wavelength of absorption.

b) **Differential Scanning Calorimetry (DSC)**
Differential scanning calorimetry was performed by using DSC-60. The instrument comprised of calorimeter (DSC 60), flow controller (FCL 60), thermal analyzer (TA 60) and operating software TA 60 from Shimadzu Corporation, Japan. The active ingredient was placed in aluminium pan and crimped, followed by heating under nitrogen flow (30 mL/min) at a scanning rate of 5 °C min⁻¹ from 30 °C to 300 °C. Aluminium pan containing same quantity of indium was used as reference. The heat flow as a function of temperature was measured.

c) **Fourier Transform Infrared Spectroscopy (FTIR)**
Infrared spectroscopy was conducted using a Shimadzu FTIR 8300 Spectrophotometer and the spectrum was recorded in the region of 4000 to 400 cm⁻¹. The procedure consisted of dispersing the active ingredient in KBr (200 - 400 mg) and compressing into discs by applying a pressure of 5 tons for 5 min in a hydraulic press. The pellet was placed in the light path and the spectrum was obtained.

3.1.2. Analytical method development by UV spectroscopy

**Standard plot of candesartan cilexetil**
Primary stock solution i.e.1.0 mg/mL was prepared in methanol. Primary stock solution is further diluted with different media (phosphate buffer pH 6.8 and pH 7.4) to get stock solution i.e. 100.0 µg/mL (secondary stock solution). Concentrations of 4.0, 8.0, 12.0, 16.0, 20.0 and 24.0 µg/mL were prepared from secondary stock solution in each media separately. The absorbance was measured against blank solution (respective buffer
solution) at 254.0 nm and calibration curve was plotted between concentration vs. absorbance.

### 3.1.3. Analytical method development and validation by reverse phase HPLC

Candesartan cilexetil is a cyclohexyl carbonate ester of (±)-1-hydroxyethyl 2-ethoxy-1-[p-(o-1H-tetrazol-5-yl phenyl) benzyl]-7-benzimidazole carboxylate having molecular weight of 610.67 g/mol. Cilexetil is an abbreviated name of cyclohexyloxy-carbonyloxy-ethyl moiety. Tetrazole ring has pKa value of 6.0 and ionizes 90% at physiological pH range. It is practically insoluble in water and sparingly soluble in methanol and ethanol (Gleiteret al., 2004; Harrold, 2002).

**Instrumentation and software**

Analytical and bioanalytical method was developed using HPLC system, LC2010cHT model (Shimadzu Corporation, Kyoto, Japan) equipped with low pressure quaternary gradient pump, dual wavelength UV detector utilizing deuterium lamp as a light source and High Throughput Autosampler. Chromatographic data was analyzed using LC solution v.1.24 SP1 software. The maximum absorbance observed at wavelength 254 nm by UV spectroscopy was selected for HPLC studies.

**Initial separation conditions**

- **Stationary phase**: Grace Vydac® C18 (250 × 4.6 mm, 5µ)
- **Mobile phase**: 20.0 mM ammonium acetate buffer (pH 4.5): acetonitrile (40:60% v/v)
- **Flow rate**: 1.0 mL/min
- **Detection wavelength**: 254.0 nm
- **Injection volume**: 25.0 µL
- **Column oven temperature**: 25.0 °C
- **Auto sampler temperature**: 4.0 °C

20.0 mM ammonium acetate buffer was prepared by dissolving 1.541 g of ammonium acetate in 1000.0 mL of mille Q water and pH was adjusted to 4.5 using glacial acetic acid. The standard solution of candesartan cilexetil (10.0 µg/mL) was injected with above conditions.
Method validation
Method validation ensures performance of the analytical method in terms of specificity and reproducibility. Method validation includes all of the procedures to demonstrate that a particular method is reliable and reproducible for its intended use (ICH Q2 R1 guidelines).

**Linearity**
Linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration of the analyte in the sample. Series of calibration concentrations were prepared ranging from 0.1 to 25.0 µg/mL of candesartan cilexetil in the mobile phase mixture and injected with optimized chromatographic conditions to record the chromatogram. Calibration curve was plotted between concentration and peak area. Correlation coefficient and regression equation were derived by fitting the data into linear regression model.

**Accuracy**
Accuracy of a developed analytical method describes the closeness of the test result obtained by the method to the true/reference value. Accuracy of the developed method was performed by recovery studies. Known amounts of the standard drug in three different concentrations were injected in triplicate and recovery of the drug is calculated.

**Precision**

*Repeatability*
Repeatability of an analytical method assesses the precision during single analytical run and between the run. It is also termed as inter-batch precision and it measures the precision with time. A solution of 10.0 µg/mL injected at three different intervals and percentage relative standard deviation was calculated.

*Intermediate precision*
It measures long term variability in measurements. It is expressed as the percent relative standard deviation for a statistically significant number of samples. It measures deviation of results from different operators, different instruments and reagents from different
suppliers. It is determined by recording the chromatogram of a standard solution in between days.

**Limit of detection (LOD) and Limit of quantification (LOQ)**
Detection limit of an analytical method is the ability to detect the lowest concentration of analyte i.e. three time peak area to the baseline noise. LOQ was determined as the lowest concentration of analyte that can be analyzed quantitatively with acceptable precision and accuracy.

**Robustness**
It assesses the capacity of a method in producing reproducible results by small deliberate changes in the optimized method. To determine the robustness, method parameters such as pH (± 0.2) of mobile phase, flow rate (± 10%), column temperature and detection wavelength (± 5 nm) are varied and influence of these changes will be evaluated.

### 3.1.4. Bioanalytical method development and validation of candesartan by HPLC
Candesartan cilexetil, non-peptide ester prodrug rapidly and completely hydrolyzes to candesartan during the absorption which has angiotensin II (Type 1, AT1) receptor antagonistic activity. Hence development of bioanalytical method for candesartan, an active metabolite of candesartan cilexetil would be useful for pharmacokinetic studies. Candesartan is white crystalline powder with molecular weight of 440.0 g/mol. Chemical name of candesartan is 2-ethoxy-1-[[2-(1H-tetra-zol-5-yl)biphenyl-4-yl]methyl]-1H-benzimidazole-7-carboxylic acid. It contains two acidic functional groups i.e. tetrazole and carboxylic group with pKa of 5.3 (Gleiter et al., 2004; Moffat et al., 2004).

Previously reported bioanalytical methods for candesartan are listed in Table 3.1. From the listed methods, it is clear that most of the methods are based on mass spectroscopy which is expensive. Peepliwal et al. (2010) developed bioanalytical method using HPLC-UV, but the method suffers from high processing volume i.e. 500.0 µL which is undesirable for pharmacokinetic studies in rats. Hence, in the present study an attempt was made to develop a simple method using liquid-liquid extraction technique with a processing volume of 200.0 µL of plasma.
Collection of rat plasma

Rat blood was collected through retro orbital-vein using heparinized capillary tube and plasma was separated by centrifuging at 10,000 rpm for 10.0 min. Supernatant was separated from the blood and stored at -70 °C until analysis.

Selection of an internal standard (IS)

Selection of IS is one of the important requirements during bioanalytical method development for quantification of drug from the biological matrix in order to evaluate the extraction efficiency. Generally selection of IS is based on structural similarities, chemical structure, solubility and polarity characteristics of the primary analyte. In the present study, Losartan was selected as an IS which has the similar chemical structure to that of candesartan and its peak was well resolved from analyte peak.

Table 3.1 Bioanalytical methods of candesartan reported in the literature

<table>
<thead>
<tr>
<th>Author</th>
<th>Matrix</th>
<th>Sample pretreatment</th>
<th>Run time</th>
<th>Limit of quantification (ng/ml)</th>
<th>Detection</th>
<th>Processing volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peepliwal et al., 2010</td>
<td>Human plasma</td>
<td>Protein precipitation</td>
<td>8.0</td>
<td>50.00</td>
<td>UV</td>
<td>500.0</td>
</tr>
<tr>
<td>Stenhoff et al., 1999</td>
<td>Human plasma &amp; Urine</td>
<td>Liquid-liquid extraction</td>
<td>25.0</td>
<td>0.1</td>
<td>Fluorescence</td>
<td>500.0</td>
</tr>
<tr>
<td>Leviet et al., 2009</td>
<td>Human plasma &amp; Urine</td>
<td>On line Solid phase extraction</td>
<td>4.5</td>
<td>1.00</td>
<td>MS</td>
<td>50.0</td>
</tr>
<tr>
<td>Erk, 2003</td>
<td>Human plasma</td>
<td>Protein precipitation</td>
<td>10.0</td>
<td>10.00</td>
<td>PDA</td>
<td>1000.0</td>
</tr>
<tr>
<td>Karra et al., 2012</td>
<td>Human plasma</td>
<td>Solid phase extraction</td>
<td>2.7</td>
<td>15.00</td>
<td>MS</td>
<td>250.0</td>
</tr>
<tr>
<td>Zhanget al., 2012</td>
<td>Rat plasma</td>
<td>Liquid-liquid extraction</td>
<td>NA</td>
<td>50.00</td>
<td>MS</td>
<td>25.0</td>
</tr>
</tbody>
</table>
### Table 3.1: Comparative Results of Different Extraction Techniques

<table>
<thead>
<tr>
<th>Study</th>
<th>Matrix</th>
<th>Extraction Method</th>
<th>LLOQ (µg/mL)</th>
<th>LOD (µg/mL)</th>
<th>detector</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prajapati et al., 2011</td>
<td>Human plasma</td>
<td>Protein precipitation</td>
<td>3.5</td>
<td>1.298</td>
<td>MS</td>
<td>100.0</td>
</tr>
<tr>
<td>Bharati et al., 2012</td>
<td>Human plasma</td>
<td>Liquid-liquid extraction</td>
<td>2.5</td>
<td>1.00</td>
<td>MS</td>
<td>100.0</td>
</tr>
</tbody>
</table>

NA-Not available, MS-Mass spectroscopy, PDA-Photodiode array

---

**Optimization of extraction procedure**

Various experiments were carried out to optimize the extraction of the drug from rat plasma with consistent recovery and devoid of endogenous interferences at the retention time of candesartan and IS. Protein precipitation method was attempted initially but drug recovery was found to be less. Later, an attempt was made by adopting liquid-liquid extraction procedure to recover the drug from the plasma. Liquid-liquid extraction method using tert-butyl methyl ether (MTBE) was adopted to extract candesartan from the rat plasma. As the recovery is less, mixture of organic solvents i.e. MTBE and dichloromethane was attempted.

**Optimization of chromatographic conditions**

Chromatographic separation is greatly affected by the composition and ratio of mobile phase delivered to the column. Methanol was used as organic phase in bioanalysis as acetonitrile exhibited less solubilization capacity for the drug. Ion pair chromatographic method was adopted to retain candesartan on C18 column. Candesartan is a weak acid with (acidic and basic functional groups) a pKa of 5.3 undergoes ionization in the pH range of 3-8. Silica bonded C18 columns are also stable in this pH range and hence results in weak retention. In ion pair chromatography, ionized organic compound will form complex with ion pair reagent to form neutral compound so that lipophilicity will increase and thereby retention in C18 column. Since candesartan is acidic in nature, tetra butyl ammonium hydroxide was used as basic ion pair reagent to form neutral complex. Methanol and 10.0 mM ammonium acetate buffer of pH 4.0 (pH adjusted using glacial acetic acid) was used as mobile phase. Mobile phase was filtered through 0.22 µm membrane filters with filtration assembly equipped with vacuum and degassed using sonicator prior to use. Column temperature and sample temperature were maintained at
25.0 °C and 4.0 °C respectively. Mobile phase delivered isocratically at a flow rate of 1.0 mL/min and eluent was monitored at 254.0 nm.

Sample extraction procedure
Plasma separated from rat blood stored at -70 °C (Sanyo ultra-low temperature freezer, UK) thawed before analysis. To 180.0 µL of blank plasma in polypropylene tube, 20.0 µL of working solution of drug was added and vortexed for 90 s. To this 20.0 µL of internal standard was added and vortexed for 90 s. To this 200.0 µL, 2.0% orthophosphoric acid was added and vortexed for 120 s. After drug extraction from the plasma, 200.0 µL of 5 mM TBAH solution of pH 4.0 was added and vortexed for 120 s to form ion pair complex with candesartan. The ion pair complex was extracted into 2.5 mL of organic solvent mixture composed of tert-butyl methyl ether and dichloromethane (4:1) and vortexed for 3.0 min to ensure complete drug extraction into the organic phase. The tubes were centrifuged (Remi Equipments Ltd., Mumbai, India) at 10,000 rpm for 10.0 min to separate the plasma components from the drug containing layer. Supernatant (2.3 mL) was separated into glass tubes and evaporated to dryness under a stream of nitrogen (TurboVap® LV, Caliper life sciences, Hopkinton, MA, USA) at 50 °C, 15 psi for 10.0 min. The dried residue was reconstituted in 100.0 µL of mobile phase mixture by vortexing for 2.0 min. The sample was transferred into vial insert and 50.0 µL was injected into the chromatographic system.

Bioanalytical method validation (FDA guidelines)
Preparation of calibration curve and quality control samples
Primary stock solution of candesartan (1.0 mg/mL) and IS (1.0 mg/mL) was prepared in methanol and stored at 2 – 8 °C when not in use. Working solutions of candesartan and IS (100.0 µg/mL) were prepared by diluting the primary stock solutions with methanol. From the working solution of candesartan, spiking stock solutions were prepared in methanol in the concentration range of 500.0 to 25,000.0 ng/mL. The quality control (QC) standards i.e. low quality control (LQC), middle quality control (MQC) and high quality control (HQC) spiking stock solutions were prepared by serial dilution from the stock solution to 1500.0, 7000.0 and 22000.0 ng/mL spiking stock solutions respectively. Calibration standards were prepared by spiking 20.0 µL of previously prepared working solutions in 180.0 µL of blank rat plasma to achieve calibration standards equivalent to
50.0, 100.0, 200.0, 400.0, 800.0, 1200.0, 1600.0, 2000.0 and 2500.0 ng/mL. Similarly, QCs were prepared from the spiking stock solutions (i.e. 1500.0, 7000.0 and 22000.0 ng/mL) to get concentrations of 150.0, 700.0 and 2200.0 ng/mL as LQC, MQC and HQC.

**Selectivity**

Selectivity is generally defined as the lack of interfering peaks at the retention times of assayed drug and internal standard in the chromatograms. It is recommended that selectivity of the method should be established with respect to endogenous substances, metabolites and known degradation products. Interferences which are likely to be present in small quantities may adversely affect the quantitation of unknown samples at concentration approaching the limit of quantitation. Selectivity of the assay was investigated by processing and analyzing blanks prepared from six independent lots of control plasma along with six extracted LOQ-QC samples. The method is selective if the response of interfering peaks at the retention time of drug is less than 20% of mean response of six extracted LOQ-QC samples.

**Recovery**

It is the ability of an extraction procedure to recover the drug from the matrix. Recovery is assessed by comparing the mean peak areas of three extracted LQC, MQC and HQC samples to mean peak areas of three neat reference solutions (unextracted). Recovery was calculated by comparing the mean peak area of an extracted sample \( (n = 6) \) to the one obtained after the direct injection of a solution with the same drug concentration.

\[
\text{Recovery} = \frac{\text{Mean peak area response of extracted samples at LQC, MQC, HQC}}{\text{Mean peak area response of unextracted samples at LQC, MQC, HQC}}
\]

**Accuracy and precision**

Both accuracy and precision (intra- and inter-day precisions) were determined by spiking the each QC samples of calibration curve. Six samples of each concentration were injected on three different days for inter day precision and within the same day for repeatability. The accuracy (%bias) was calculated from the mean value of observed concentration (Cobs) and the nominal concentration (Cnom) as follows

\[
\text{Accuracy (\% bias)} = \frac{(\text{Cobs})-(\text{Cnom})}{(\text{Cnom})} \times 100
\]
The percent coefficient of variation, \(\%CV\) was calculated from the observed concentrations as follows:
\[
\% CV = \frac{\text{Standard deviation (SD)}}{\text{Cobs}} \times 100
\]

The accuracy determined at each concentration level must be within ±15% of the respective nominal value except at LOQ-QC where it must be within ±20% of the nominal value. The precision around the mean value must not exceed 15% of the CV except for LOQ-QC where it must not exceed 20% of the CV.

**Stability studies in the matrix**

The drug stability in a biological fluid is a function of the storage conditions, the chemical properties of the drug and the matrix. Stability of the drug in rat plasma was assessed by storing the spiked plasma samples at room temperature (bench top), freezer (freeze-thaw stability) and long term stability (storing the samples at -70 °C for three months). These stability studies were performed by replicate analysis of LQC and HQC samples. Bench top stability was carried out with LQC and HQC samples in the biological matrix which were withdrawn and thawed at room temperature and kept unprocessed for 7 h (stability samples). After 7 h, fresh calibration sample was prepared with one set of low and high QC samples (comparison samples). Freeze thaw stability in plasma was assessed by analyzing six replicates of LQC and HQC samples after three freeze and thaw cycles. Samples were stored at the intended storage temperature for 24 h and thawed unassisted at room temperature. Long term stability studies were carried out with six replicates of LQC and HQC in the biological matrix which were withdrawn from deep freezer (-70 °C) after 30 days and thawed at room temperature (stability samples). Fresh calibration was prepared with six replicates of low and high QC samples (comparison samples). In all the above stability studies, both comparison samples and stability samples were processed and analyzed in a single run.

### 3.1.5. Results and discussion

#### 3.1.5.1. Identification of the drug substance

**a) UV spectrum**

A solution of 10.0 µg/mL of candesartan cilexetil in methanol was scanned in the range of 200 - 400 nm using methanol as blank. The maximum wavelength of absorption \((\lambda_{max})\) was found to be 254.0 nm.
b) Differential Scanning Calorimetry (DSC)

Pure drug showed a sharp endotherm at 163.8 °C corresponding to its melting point/transition temperature. This is in accordance with Certificate of Analysis (COA). Thermogram of candesartan cilexetil was depicted in Fig. 3.1.

![DSC thermogram of candesartan cilexetil](image)

Fig. 3.1. DSC thermogram of candesartan cilexetil

c) Fourier Transform Infrared Spectroscopy (FTIR)

The characteristic infrared absorbance peaks were observed at 750, 1040, 1080, 1245, 1280, 1355, 1430, 1550, 1730, 1750 and 2860 cm\(^{-1}\). These peaks were in accordance with the previously reported literature (Yatendra et al., 2005). An Infrared spectrum is presented in Fig. 3.2.
3.1.5.2. Analytical method development by UV spectroscopy

*Standard plot of candesartan cilexetil by UV spectroscopy*

Calibration curve (CC) was plotted by measuring absorbance of candesartan cilexetil containing solutions at 254 nm. CC was plotted in various buffers like pH 6.8 phosphate buffers, pH 7.4 phosphate buffer (Fig. 3.3). Standard plots were prepared in various media showed good linearity and with acceptable regression values.

![Fig. 3.3. Standard plot of candesartan cilexetil in phosphate buffer A) pH-6.8, B) pH 7.4 by UV-visible spectrophotometer](image-url)
3.1.5.3. Analytical method development and validation by reverse phase HPLC method

Reverse phase HPLC method with UV detection was developed for estimation of candesartan cilexetil for its formulation evaluations like drug content, in vitro drug release and stability studies. Typical standard chromatogram is shown in Fig. 3.4.

*Optimized chromatographic conditions for the analytical method*

- **Stationary phase**: Grace Vydac C$_{18}$ column (250 × 4.6 mm, 5µ)
- **Mobile phase**: 20.0 mM ammonium acetate buffer pH 4.5:acetonitrile (45:55% v/v)
- **Flow rate**: 1.0 mL/min
- **Detection wavelength**: 254.0 nm
- **Injection volume**: 25.0 µL
- **Column oven temperature**: 25.0 °C
- **Auto sampler temperature**: 4.0 °C

The standard solution i.e. 10.0 µg/mL of candesartan cilexetil was injected for analyzing retention time. Retention time was found to be about 5.8 min with 40:60% v/v. As the elution is faster, buffer ratio increased to 45%. Retention time was found to be about 8.3 min with the mobile phase delivered at 45:55 ratios, which is due to reduction in percentage of organic solvent, decreased the solubility of the analyte in the mobile phase. Strength of the phosphate buffer does not have significant effect on retention time of the analyte. Hence, in the present method 20.0 mM pH 4.5 ammonium acetate buffer was used as mobile phase.

![Typical chromatogram of candesartan cilexetil](image-url)
Linearity
Proposed method was found to be linear in the range of 0.1 – 25.0 µg/mL. Linearity is generally reported by the coefficient determination \( (R^2) \) value. In the present method, \( R^2 \) was found to be 0.999 indicating that the proposed method was linear. Calibration curve is shown in Fig. 3.5. The slope and intercept were used to determine the unknown concentration of the test solution (results of analytical method validation are shown in Table 3.2).

**Fig. 3.5.** Calibration curve of candesartan cilexetil by HPLC-UV method

**LOD and LOQ**
LOD and LOQ were calculated based on the standard deviation of the response and slope.

\[
\text{LOD} = 3.3 \times \frac{\text{SD}}{\text{S}}
\]

\[
\text{LOQ} = 10 \times \frac{\text{SD}}{\text{S}}
\]

where SD = Standard deviation of blank response; S = Slope of the calibration curve;

LOD and LOQ were found to be 0.01 and 0.03 µg/mL respectively. This indicates that the developed method is sensitive for the quantification of candesartan cilexetil.

**Table 3.2** Summary of analytical method validation of candesartan cilexetil by HPLC-UV method

<table>
<thead>
<tr>
<th>Validation parameter</th>
<th>Results</th>
<th>Acceptance criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accuracy (%Mean ± SD)</td>
<td>100.5 ± 1.12</td>
<td>98 – 102</td>
</tr>
<tr>
<td>Repeatability precision (%RSD)</td>
<td>0.42</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Intermediate precision (%RSD)</td>
<td>0.89</td>
<td>&lt; 2</td>
</tr>
<tr>
<td>Linearity ( (R^2) )</td>
<td>0.999</td>
<td>&gt; 0.990</td>
</tr>
<tr>
<td>LOD (µg/mL)</td>
<td>0.01</td>
<td>S/N ratio should be 3:1</td>
</tr>
<tr>
<td>LOQ (µg/mL)</td>
<td>0.03</td>
<td>S/N ratio should be 10:1</td>
</tr>
</tbody>
</table>
3.1.5.4. Bioanalytical method development and validation of candesartan by HPLC

A sensitive and selective method for the estimation of drug in biological matrix is very much essential to evaluate the efficiency of the developed dosage form in vivo. Pharmacokinetic profile of pure drug can be compared against the developed formulation by estimating the drug concentration in the biological matrix.

Method development

Very weak retention for candesartan was observed with standard reverse phase chromatographic conditions. This could be because of the acidic functional groups (carboxyl and tetrazole group) present in candesartan with pKa of 5.3 (Gleiter et al., 2004). The carboxyl and amino functional groups present in the compound are susceptible to deprotonation and ionization respectively at the pH range of 3.0-8.0 (the pH range used in standard reverse phase chromatographic conditions). This leads to increased hydrophilicity of the candesartan and weak retention in a reverse phase column. In order to improve the retention on C18 column, ion pair method was utilized to reduce the ionization. Mobile phase containing methanol and 5 mM TBAH ammonium acetate buffer (pH 4.0) has shown good retention. This implies that at pH 4.0, weakly acidic candesartan ionizes to form a stable ion pair with TBAH (Fig. 3.6). This complex formation resulted in improved lipophilicity of the compound that increases the retention time in C18 column.

![Complex formation of candesartan with TBAH](image)

**Fig. 3.6.** Complex formation of candesartan with TBAH

However, recovery was less when the ion-pair reagent was added to the mobile phase which may be due to the minimal exposure of candesartan with TBAH to form a stable
ion pair. In order to improve the recovery, test solutions were prepared in a mixture of methanol and 5 mM TBAH of pH 4.0 to form an ion pair complex externally. This attempt significantly improved the recovery. This may be attributed to the improved hydrophobicity of the ion pair. To our surprise, further enhancement of TBAH to 10 mM has resulted in peak broadening. Considering this, ion pair complex was formed externally during extraction by adding 200.0 µL of 5 mM TBAH solution. However, enhancement in the amount of TBAH to 400.0 µL did not result in enhanced recovery.

At alkalence plasma pH, it was speculated that carboxylic functional group of candesartan deprotonates to form complex with protein. Acidification of plasma can destabilize this complex. Hence, plasma was acidified using 200.0 µL of 2.0% orthophosphoric acid to protonate the carboxylic acid functionality of candesartan and to increase its affinity towards organic solvent used for extraction.

**Optimized chromatographic conditions**

- **Stationary phase**: Grace Vydac C18 column (250.0 × 4.6 mm, 5 µ)
- **Mobile phase**: 10.0 mM ammonium acetate buffer pH 4.0: methanol (47:53% v/v)
- **Flow rate**: 1.0 mL/min
- **Detection wavelength**: 254.0 nm
- **Injection volume**: 50.0 µL
- **Column oven temperature**: 25.0 °C
- **Auto sampler temperature**: 4.0 °C

**Bioanalytical method validation**

**Selectivity**

Pooled blank plasma samples (n = 6) were processed with and without internal standard to detect interferences from endogenous matrix at the retention time of the analyte and internal standard. Candesartan and IS are well separated from plasma proteins and devoid of interferences under optimized extraction procedure and chromatographic condition. Chromatograms of blank plasma and plasma spiked with drug and IS were shown in Fig. 3.7 and 3.8.
Linearity

Concentration vs. peak area ratio of candesartan to the IS was plotted to check the linearity. Calibration curve is constructed in the concentration range of 50.0 – 2500.0 ng/mL and found to be linear with coefficient determination ($R^2$) of 0.995. Calibration curve is shown in Fig. 3.9.

![Fig. 3.7. Representative chromatogram of blank rat plasma](image1)

![Fig. 3.8. Representative chromatogram of rat plasma spiked with candesartan](image2)
Fig. 3.9. Calibration curve of candesartan in rat plasma by HPLC-UV method

Lower limit of quantitation (LLOQ)
The LLOQ of 50.0 ng/mL was selected in the present method. The response of analyte was more than 5 times of the blank response. Accuracy (80-120%) and precision (20%) was carried out and it was found to be within the acceptable limits.

Table 3.3 Summary of bioanalytical method validation of candesartan

<table>
<thead>
<tr>
<th>Experimental parameter</th>
<th>Validation report</th>
<th>Acceptance limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibration range (Coefficient of determination)</td>
<td>50.0 – 2500.0 ng/mL (R² = 0.995)</td>
<td>&gt; 0.98 with consistency</td>
</tr>
<tr>
<td>Intra batch precision (% CV)</td>
<td>1.47 – 8.25</td>
<td>±15% dev. from nominal conc. except at LLOQ ± 20% dev.</td>
</tr>
<tr>
<td>Intra batch accuracy (%)</td>
<td>93.2 – 102.2</td>
<td>85-115% except the LLOQ (80-120%)</td>
</tr>
<tr>
<td>Inter batch precision (% CV)</td>
<td>3.02 – 7.97</td>
<td>±15% dev. from nominal conc. except at LLOQ ± 20% dev.</td>
</tr>
<tr>
<td>Inter batch accuracy (%)</td>
<td>97.42 – 105.26</td>
<td>85–115% except the LLOQ (80-120 %)</td>
</tr>
<tr>
<td>% Relative recovery (Mean ± SD)</td>
<td>Candesartan – 69.8 ± 4.3</td>
<td>Consistent recovery</td>
</tr>
<tr>
<td></td>
<td>Losartan – 73.2 ± 3.5</td>
<td></td>
</tr>
</tbody>
</table>
### Stability

<table>
<thead>
<tr>
<th>Condition</th>
<th>Range (%)</th>
<th>Acceptance Limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freeze thaw (-70 °C)</td>
<td>92.42 – 96.82</td>
<td>Mean % change should be within 85 – 115%</td>
</tr>
<tr>
<td>Bench top stability (8 h)</td>
<td>98.21 – 101.23</td>
<td>Mean % change should be within 85 – 115%</td>
</tr>
<tr>
<td>Long term stability (30 days, -70 °C)</td>
<td>95.86 – 98.21</td>
<td>Mean % change should be within 85 – 115%</td>
</tr>
</tbody>
</table>

### Accuracy and precision of QC samples

The accuracy and precision of low, medium and high quality control samples (n = 6) were determined by analyzing between intra-day and inter-day. The results of method validation are presented in Table 3.3. The accuracy determined at each concentration level was within the acceptance limit i.e. ± 15% and at LOQ-QC it was within the acceptance limit i.e. ± 20% of the nominal value. The precision determined by calculating %CV value. The %CV for LOQ was within 20%, and for remaining all QC samples, it was within 15% limit.

### Recovery

The extraction recovery (Mean ± SD) for candesartan and IS was found to be 69.8 ± 4.3% and 73.2% ± 3.5% respectively.

### Stability studies

There were no significant changes in concentration and it can be concluded that candesartan is stable at different storage conditions. The percentage change was within the limit.
3.2. Analytical and bioanalytical method development for eprosartan mesylate

3.2.1. Identification of the drug substance

a) UV spectroscopy
According to the procedure described in 3.1.1

b) Differential Scanning Calorimetry (DSC)
According to the procedure described in 3.1.1

c) Fourier Transform Infrared Spectroscopy (FTIR)
According to the procedure described in 3.1.1

3.2.2. Analytical method development by UV spectroscopy

Standard plot of eprosartan mesylate
Primary stock solution i.e. 1.0 mg/mL of eprosartan mesylate was prepared in methanol. Primary stock solution is further diluted with different media (phosphate buffer pH 6.8 and phosphate buffer pH 7.4) to get stock solution i.e. 100.0 µg/mL (secondary stock solution). Concentrations of 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, 14.0, 16.0, 18.0 and 20.0 µg/mL were prepared from secondary stock solution in each media. The absorbance was measured against blank solution (respective buffer solution). Calibration curve was plotted between concentration and absorbance by recording absorbance at 232.0 nm.

3.2.3. Analytical method development and validation by reverse phase HPLC
Eprosartan mesylate is a non-biphenyl, non-tetrazole angiotensin II antagonist having molecular weight of 520.62 g/mol. The chemical name of eprosartan mesylate is (E)-α-[[2-butyl-1-[(4-carboxyphenyl) methyl]-1H-imidazol-5-yl] methyl-ene] 2-thiophene propanoic acid monomethanesulfonate. The pKa of carboxylic group and imidazole functional groups of eprosartan are 3 - 4 and 6.8 respectively. Primarily, the carboxylic group has the tendency to get ionized at physiological pH. Eprosartan mesylate is insoluble in water and freely soluble in methanol (Harrold, 2002; O'Neil, 2006).
Instrumentation and software

Analytical and bioanalytical method was developed using HPLC system, LC-2010cHT model (Shimadzu Corporation, Kyoto, Japan) equipped with low pressure quaternary gradient pump, dual wavelength UV detector and High Throughput Autosampler. Chromatographic data was analyzed using LC solution v.1.24 SP1 software.

Initial separation conditions

From primary stock solution 10.0 µg/mL of eprosartan mesylate solution was prepared using methanol as a solvent. Acetonitrile was selected as organic phase due to its favorable UV transmittance and low back pressure.

Stationary phase: Hibar®C18 (250 × 4.6 mm, 5µ)
Mobile phase: 10.0 mM phosphate buffer pH 3.0:acetonitrile (50:50% v/v)
Flow rate: 1.0 mL/min
Detection wavelength: 232.0 nm
Injection volume: 25.0 µL
Column oven temperature: 25.0 °C
Auto sampler temperature: 4.0 °C

10.0 mM phosphate buffer was prepared by dissolving 1.3609 g of potassium dihydrogen phosphate in 1000.0 mL of milli Q water and pH was adjusted to 3.0 using orthophosphoric acid.

Method validation (ICH Q2R1 guidelines)

Method validation ensures performance of the analytical method in terms of specificity and reproducibility. Method validation includes all of the parameters that ensure that a particular method is reliable and reproducible for the intended use.

Linearity

Linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. Series of calibration concentrations of eprosartan mesylate were prepared ranging from 0.5 to 25.0 µg/mL in the mobile phase mixture and injected with optimized
chromatographic conditions to record the chromatogram. Calibration curve was plotted between concentration and peak area. Correlation coefficient and regression equation were derived by fitting the data into linear regression model.

**Accuracy**

Accuracy of a developed analytical method describes the closeness of the test result obtained by the method to the true/reference value. Accuracy of the method was performed by recovery studies. Known amounts of standard drug in three different concentrations were injected in triplicate and recovery of the drug was calculated.

**Precision**

**Repeatability**

Repeatability of an analytical method assesses the precision during single analytical run and between the runs. It is also termed as inter-batch precision, and measures the precision with time. A solution of 10.0 µg/mL of eprosartan mesylate was injected at three different time intervals and percentage relative standard deviation was calculated.

**Intermediate precision**

It measures long term variability in measurements. It is determined by analyzing the method with a standard solution in between days. It is expressed as the percent relative standard deviation for a statistically significant number of samples. It measures deviation of results from different operators, different instruments and reagents from different suppliers.

**Robustness**

It assesses the capacity of the method in producing reproducible results by small deliberate changes in the optimized method. To determine the robustness, method parameters such as pH (± 0.2) of mobile phase, flow rate (± 10%), column temperature, and detection wavelength (± 5 nm) are varied and their influence on retention time and peak area was evaluated.
Limit of detection (LOD) and Limit of quantification (LOQ)
Detection limit of an analytical method is the ability to detect the lowest concentration of analyte i.e. three time peak area to the baseline noise. LOQ was determined as the lowest concentration of an analyte that can be analyzed quantitatively with acceptable precision and accuracy.

3.2.4. Bioanalytical method development and validation of eprosartan mesylate by HPLC
Previously reported bioanalytical methods of eprosartan are listed in Table 3.4. So far, only Lundberg et al. (1998) and Ferreiros et al. (2006) reported HPLC-UV method for the estimation of eprosartan in human plasma. Both the methods have the disadvantage of employing high cost and relatively lengthy solid phase extraction procedure for isolation of the drug from plasma. Lundberg et al. (1998) employed commercially unavailable internal standard. Gradient elution method and high processing volume are the disadvantages associated with the method developed by Ferreiros et al. (2006). Remaining reported methods utilized mass spectroscopy which is expensive.

<table>
<thead>
<tr>
<th>Author</th>
<th>Matrix</th>
<th>Sample pretreatment</th>
<th>Run time</th>
<th>Limit of quantification (ng/ml)</th>
<th>Detection</th>
<th>Processing volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Li et al., 2007</td>
<td>Human plasma &amp; Urine</td>
<td>Protein precipitation</td>
<td>2.0</td>
<td>5.0</td>
<td>MS</td>
<td>50.0 for plasma</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>20.0 for urine</td>
</tr>
<tr>
<td>Lundberg et al., 1998</td>
<td>Human plasma</td>
<td>Solid phase extraction</td>
<td>16.0</td>
<td>10.0</td>
<td>UV</td>
<td>500.0</td>
</tr>
<tr>
<td>Ferreiros et al., 2006</td>
<td>Human plasma</td>
<td>Solid phase extraction</td>
<td>14.0</td>
<td>150</td>
<td>UV</td>
<td>1000.0</td>
</tr>
<tr>
<td>(Gradient elution)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ferreiros et al., 2007</td>
<td>Human plasma</td>
<td>Protein precipitation</td>
<td>7.3</td>
<td>13.0</td>
<td>MS</td>
<td>NA</td>
</tr>
</tbody>
</table>

Table 3.4 Earlier reported bioanalytical methods for eprosartan mesylate
The developed method is simple and economic as it involves cost effective liquid liquid extraction technique with low processing volume. The internal standard employed was also commercially available.

Preclinical studies were performed in rats, as it is well established animal model and reliable predictor of bioavailability in humans because of its physiological resemblance with humans.

**Collection of rat plasma**
Plasma was separated from the rat blood (collected through retro orbital-vein using heparinized capillary tube) by centrifuging at 10,000 rpm for 10.0 min. Supernatant was separated from the blood components and stored at -70 °C till analysis.

**Selection of an internal standard (IS)**
Selection of IS is one of the important stages in bioanalytical method development for quantification of drug from biological matrix in order to evaluate the extraction efficiency. Generally, IS selection is based on structural similarities, chemical structure, solubility and polarity characteristics of the primary analyte. In the present study, Losartan was selected as an IS which has the similar chemical structure to that of eprosartan and its peak was well resolved form eprosartan peak.

**Optimization of extraction procedure**
Various experiments were carried out to extract the drug from rat plasma with consistent recovery devoid of endogenous interferences at the retention time of eprosartan and IS. Protein precipitation method was initially attempted but drug was not recovered. Later,
an attempt was made using liquid-liquid extraction procedure to recover drug from the plasma with tert-butyl methyl ether (MTBE).

Optimization of chromatographic conditions
Chromatographic separation is greatly affected by the composition of mobile phase and their ratio delivered to the column. Methanol was used as organic phase in bioanalysis as acetonitrile resulted in interferences at retention time of the analyte. Methanol and 10.0 mM phosphate buffer pH 3.0 (pH adjusted using orthophosphoric acid) was used as mobile phase. Mobile phase was filtered through 0.22 µm membrane filters with filtration assembly equipped with vacuum and degassed using sonicator prior to use. Mobile phase was delivered isocratically at a flow rate of 1.0 mL/min and eluent was monitored at 232.0 nm.

Extraction of eprosartan mesylate from rat plasma
Plasma separated from the rat blood stored at (Sanyo ultra-low temperature freezer, UK) – 70 °C was thawed before processing. 10 µL of drug (calibration standard) solution and 10 µL of 10 µg/mL internal standard was mixed and vortexed for 90 s. 100 µL of 0.1 M HCl was added to the drug spiked plasma and vortexed for 120 s. From the aqueous phase, drug was extracted into the organic phase using 2.0 mL of MTBE by vortexing for 3.0 min. Organic phase was separated by cold centrifugation at 10,000 rpm for 10 min. Approximately 1.8 mL of supernatant was taken in a glass tube and evaporated at 50 °C under nitrogen stream at 10 - 15 psi. Dried residue was reconstituted with 150.0 µL of mobile phase and 50.0 µL of this sample was injected into HPLC.

Bioanalytical method validation (FDA guidelines)
Preparation of calibration curve and quality control samples
Primary stock solution of eprosartan mesylate (1.0 mg/mL) equivalent to eprosartan and IS (1.0 mg/mL) were prepared in methanol and stored at 2 - 8 °C when not in use. Working solutions of eprosartan and IS (100.0 µg/mL) were prepared by diluting the primary stock solutions with methanol. From the working solution of eprosartan, spiking stock solutions were prepared in methanol in the range of 250.0 to 10,000.0 ng/mL. The quality control (QC) standards i.e. low quality control (LQC), middle quality control (MQC) and high quality control (HQC), spiking stock solutions were prepared by serial
dilution from the stock solution to concentrations of 750.0, 5000.0 and 9000.0 ng/mL spiking stock solutions respectively. Calibration standards were prepared by spiking 10.0 µL of previously prepared working calibrating solutions in 90.0 µL of blank rat plasma to achieve calibration standards equivalent to 50.0, 100.0, 150.0, 200.0, 400.0, 600.0, 800.0 and 1000.0 ng/mL. Similarly, QCs were prepared from the spiking stock solutions (i.e. 750.0, 5000.0 and 9000.0 ng/mL) to get concentrations of 75.0, 500.0 and 900.0 ng/mL as LQC, MQC and HQC.

**Selectivity**
Selectivity is generally defined as the ability of a bioanalytical method to discriminate the analyte and IS from interfering components. It is recommended that selectivity of the method should be established with respect to endogenous substances, metabolites and known degradation products. Interferences which are likely to be present in small quantities may adversely affect the quantitation of unknown samples at the LOQ. Selectivity of the assay was investigated by processing and analyzing blanks prepared from six independent lots of control plasma along with six extracted LOQ-QC samples. The method is selective if the response of interfering peaks at the retention time of drug is less than 20% of mean response of six extracted LOQ-QC samples.

**Recovery**
It is the ability of extraction procedure to recover the drug from the matrix. Recovery is assessed by comparing the mean peak areas of three extracted LQC, MQC and HQC samples to mean peak areas of three neat reference solutions (unextracted). Recovery was calculated by comparing the mean peak area of an extracted sample (n = 6) to the one obtained after the direct injection of a solution with the same drug concentration.

\[
\text{Recovery} = \frac{\text{Mean peak area response of extracted samples at LQC, MQC, HQC}}{\text{Mean peak area response of unextracted samples at LQC, MQC, HQC}}
\]

**Accuracy and precision**
Both accuracy and precision (intra- and inter-day precisions) were determined by spiking each QC samples of calibration curve. Six samples of each concentration were injected on three different days for inter day precision and within the same day for repeatability.
The accuracy (%bias) was calculated from the mean value of observed concentration (Cobs) and the nominal concentration (Cnom) as follows:

\[
\text{Accuracy (% bias)} = \left[ \frac{(\text{Cobs}) - (\text{Cnom})}{\text{Cnom}} \right] \times 100
\]

The percent coefficient of variation, %CV was calculated from the observed concentrations as follows:

\[
\% \text{ CV} = \left[ \frac{\text{standard deviation (SD)}}{\text{Cobs}} \right] \times 100
\]

The accuracy determined at each concentration level must be within ± 15% of the respective nominal value except at LOQ-QC where it must be within ± 20% of the nominal value. The precision around the mean value must not exceed 15% of the CV except for LOQ-QC where it must not exceed 20% of the CV.

### Stability studies in the matrix

Stability of the drug in rat plasma was assessed by storing the spiked plasma samples at room temperature (bench top), freezer (freeze-thaw stability) and long term stability (storing the samples at -70 °C for three months). These stability studies were performed by replicate analysis of LQC and HQC samples. Bench top stability was carried out with LQC and HQC samples in the biological matrix which were withdrawn and thawed at room temperature and kept unprocessed for 7 h (stability samples). After 7 h, fresh calibration samples were prepared with one set of low and high QC samples (comparison samples). Freeze thaw stability in plasma was assessed by analyzing six replicates of LQC and HQC samples after three freeze and thaw cycles. Samples were stored at the intended storage temperature for 24 h and thawed unassisted at room temperature. Long term stability studies were carried out with six replicates of LQC and HQC in the biological matrix which were withdrawn from deep freezer (-70 °C) after 30 days and thawed at room temperature (stability samples). Fresh calibration was prepared with six replicates of low and high QC samples (comparison samples). In all the above stability studies, both comparison samples and stability samples were processed and analyzed in a single run.

### 3.2.5. Results and discussion

**Identification of the drug substance**

In order to confirm the purity of active ingredient, UV spectrum in methanol, DSC, FTIR spectra were recorded.
**a) UV spectrum**
A solution of 10.0 µg/mL in methanol was scanned in the range of 200 - 400 nm using methanol as blank. The maximum wavelength of absorption ($\lambda_{\text{max}}$) was found to be 232.0 nm.

**b) Differential Scanning Calorimetry (DSC)**
Pure drug showed a sharp endotherm at 252.4 °C corresponding to its melting point/transition temperature. This is in accordance with Certificate of Analysis (COA). Typical thermogram is shown in Fig. 3.10.

![DSC thermogram](image)

**Fig. 3.10.** DSC thermogram of eprosartan mesylate

**c) Fourier Transform Infrared Spectroscopy (FTIR)**
The characteristic infrared absorbance’s were observed at 748, 854, 1041, 1420, 1504, 1537, 1647, 1691 cm$^{-1}$. These peaks were in accordance with the reported literature. An Infrared spectrum of eprosartan mesylate is presented in Fig. 3.11.
3.2.5.2. Analytical method development by UV spectroscopy

*Standard plot of eprosartan mesylate*

Calibration curve (CC) was plotted by measuring the absorbance at 232.0 nm ($\lambda_{\text{max}}$). Calibration curve was plotted in pH 6.8 phosphate buffer and pH 7.4 phosphate buffers. Standard plots were prepared in different media showed good linearity and with acceptable regression values (Fig. 3.12).

![Calibration curve plots](image)

**Fig. 3.12.** Calibration curve in A) pH 6.8 phosphate buffer B) pH 7.4 phosphate buffer by UV spectrophotometer

3.2.5.3. Analytical method development and validation by reverse phase HPLC method

Reverse phase HPLC method with UV detection was developed for the estimation of eprosartan mesylate for its formulation evaluations like drug content, *in vitro* drug
release and stability studies. The typical standard chromatogram is shown in Fig. 3.13. Retention time was found to be 6.9 min.

![Typical chromatogram of eprosartan mesylate](image)

**Fig. 3.13.** Typical chromatogram of eprosartan mesylate

**Chromatographic conditions optimized for analytical method**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stationary phase</td>
<td>Hibar® C18 (250 × 4.6 mm, 5µ)</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>10.0 mM phosphate buffer pH 3.0:acetonitrile (60:40% v/v)</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1.0 mL/min</td>
</tr>
<tr>
<td>Detection wavelength</td>
<td>232.0 nm</td>
</tr>
<tr>
<td>Injection volume</td>
<td>25.0 µL</td>
</tr>
<tr>
<td>Column oven temperature</td>
<td>25.0 °C</td>
</tr>
<tr>
<td>Auto sampler temperature</td>
<td>4.0 °C</td>
</tr>
</tbody>
</table>

Standard solution of eprosartan mesylate, 10.0 µg/mL, was injected for analyzing the retention time. The retention time was found to be about 5.0 min with 50:50% v/v mobile phase. As the elution is faster, buffer ratio increased to 60% and elution was achieved with retention time of 6.9 min. This is due to reduction in percentage of organic solvent that decreased the solubility of the analyte in the mobile phase. The strength of phosphate buffer did not show significant effect on the retention time of eprosartan mesylate. Hence, in the present method 10.0 mM phosphate buffer of pH 3.0 was selected as mobile phase.
Linearity

Proposed method was found to be linear in the range of 0.5 – 25.0 µg/mL. Linearity is generally reported by the coefficient determination ($R^2$) value. In the present method, $R^2$ was found to be 0.999 which indicates that the proposed method was linear. Calibration curve is shown in Fig. 3.14. The slope and intercept were used to determine the unknown concentration of the test solution (Results of analytical method validation are presented in Table 3.5).

![Calibration curve of eprosartan mesylate by HPLC-UV method](image)

**Fig. 3.14.** Calibration curve of eprosartan mesylate by HPLC-UV method

**LOD and LOQ**

LOD and LOQ were calculated based on the standard deviation of the response and slope.

$LOD = 3.3 \times SD/S$

$LOQ = 10 \times SD/S$ where SD = Standard deviation of blank response; S = Slope of the calibration curve

LOD and LOQ were found to be 0.01 and 0.03 µg/mL respectively. This indicates that the developed method is sensitive for the quantification of eprosartan mesylate.

**Table 3.5** Summary of analytical method validation of eprosartan mesylate by HPLC-UV method

<table>
<thead>
<tr>
<th>Validation parameter</th>
<th>Results</th>
<th>Acceptance criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accuracy (% Mean ± SD)</td>
<td>99.8 ± 1.6</td>
<td>98 – 102</td>
</tr>
<tr>
<td>Repeatability precision (% RSD)</td>
<td>0.35</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Intermediate precision (% RSD)</td>
<td>0.72</td>
<td>&lt; 2</td>
</tr>
<tr>
<td>Linearity ($R^2$)</td>
<td>0.999</td>
<td>&gt; 0.990</td>
</tr>
<tr>
<td>LOD (µg/mL)</td>
<td>0.01</td>
<td>S/N ratio should be 3:1</td>
</tr>
<tr>
<td>LOQ (µg/mL)</td>
<td>0.03</td>
<td>S/N ratio should be 10:1</td>
</tr>
</tbody>
</table>
3.2.5.4. Bioanalytical method development and validation of eprosartan mesylate by HPLC

A sensitive and selective method for the estimation of drug in biological matrix is very much essential to evaluate the efficiency of developed dosage form in vivo. Validation of a developed method is mandatory in order to ensure the accurate results when applied for routine analysis. Pharmacokinetic profile of pure drug can be compared against the developed formulation by analyzing the drug concentration in the biological matrix.

Method development

Since protein precipitation method resulted in poor recovery, liquid-liquid extraction method was attempted. Though acetonitrile is widely used organic solvent due to its UV transmittance and low back pressure, it did not result in adequate retention of analyte and failed to provide sufficient resolution between the drug and IS. Hence methanol was used as organic solvent for the method development. Adequate retention with best resolution between the drug and IS was achieved with 43% potassium dihydrogen phosphate buffer of pH 3.0 and 57% methanol as mobile phase composition delivered isocratically. Protein precipitation method with various reagents such as acetonitrile, methanol, mixture of acetonitrile and methanol, chilled acetonitrile and perchloric acid was attempted initially to extract the drug from the plasma. These methods were unable to extract the drug from the matrix which might be due to polar nature of the molecule (99% protein bound drug). Liquid-liquid extraction using MTBE as an extracting solvent also failed to yield good recovery. Acidification of the plasma with 0.1N HCl enhanced the extraction recovery. Deprotonation of phenyllic carboxylic group at plasma pH imparts hydrophilicity to the analyte which results in poor extraction into organic solvent. Thus the addition of 100.0 μL of 0.1 M HCl protonates the carboxylic acid functionality of eprosartan and destabilizes the complex with plasma proteins and increases its affinity towards organic solvent used for extraction. MTBE was selected as extracting solvent after evaluating the recoveries and noise in the chromatogram using n-hexane, dichloromethane, combination of MTBE and dichloromethane. Based on the solubility of the analyte, mixture of methanol and potassium dihydrogen phosphate buffer of pH 3.0 (50:50; 150.0 μL) was finalized as reconstitution diluent.
Optimized chromatographic conditions

Stationary phase: Hibar® C18 (250 × 4.6 mm, 5µ)

Mobile phase: 10.0 mM phosphate buffer pH 3.0: methanol (43:57% v/v)

Flow rate: 1 mL/min

Detection wavelength: 232.0 nm

Injection volume: 50.0 µL

Column oven temperature: 25.0 °C

Auto sampler temperature: 4.0 °C

Bioanalytical method validation

Selectivity

Pooled blank plasma samples (n = 6) were processed with and without internal standard and to detect interferences from endogenous matrix at the retention time of the analyte and internal standard. Eprosartan and IS are well separated from the plasma proteins devoid of interferences using optimized extraction procedure and chromatographic conditions. Chromatograms of blank plasma and plasma spiked with drug and IS were shown in Fig. 3.15 and 3.16.

![Chromatogram of blank plasma](image)

**Fig. 3.15.** Chromatogram of blank plasma
Fig. 3.16. Chromatogram of plasma spiked with drug and IS

**Linearity**

Concentration vs. peak area ratio of eprosartan mesylate to the IS was plotted to check the linearity. Calibration curve is constructed for concentrations between 50 – 1000 ng/mL and found to be linear with coefficient determination ($R^2$) of 0.998. Calibration curve is shown in Fig. 3.17.

![Calibration curve](image)

Fig. 3.17. Calibration curve in rat plasma by HPLC-UV method

**Lower limit of quantitation (LLOQ)**

LLOQ of 50.0 ng/mL was selected as the response of analyte was more than 5 times of the blank response. Accuracy (80 - 120%) and precision (20%) were found to be within the acceptable limits.
### Table 3.6 Summary of bioanalytical method validation of eprosartan mesylate

<table>
<thead>
<tr>
<th>Experimental parameter</th>
<th>Validation report</th>
<th>Acceptance limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibration range (Coefficient of determination)</td>
<td>50 – 1000 ng/mL (R² = 0.998)</td>
<td>&gt; 0.98 with consistency</td>
</tr>
<tr>
<td>Intra batch precision (% CV)</td>
<td>1.45 – 9.63</td>
<td>±15% dev. from nominal conc. except at LLOQ ± 20% dev.</td>
</tr>
<tr>
<td>Intra batch accuracy (%)</td>
<td>95.62 – 104.67</td>
<td>85-115% except the LLOQ (80-120%)</td>
</tr>
<tr>
<td>Inter batch precision (% CV)</td>
<td>3.42 – 10.43</td>
<td>±15% dev. from nominal conc. except at LLOQ ± 20% dev.</td>
</tr>
<tr>
<td>Inter batch accuracy (%)</td>
<td>91.34 – 106.32</td>
<td>85–115% except the LLOQ (80-120 %)</td>
</tr>
<tr>
<td>% Relative recovery (Mean ± SD)</td>
<td>Eprosartan -72.4 ± 4.9% Losartan - 66.6 ± 4.1%</td>
<td>Consistent recovery</td>
</tr>
<tr>
<td>Stability</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Freeze thaw (-70 ºC)</td>
<td>93.55 – 97.42</td>
<td>Mean % change should be within 85 – 115%</td>
</tr>
<tr>
<td>Bench top stability (8 h)</td>
<td>97.23 – 100.9</td>
<td>Mean % change should be within 85 – 115%</td>
</tr>
<tr>
<td>Long term stability (30 days, -70 ºC)</td>
<td>95.12 – 99.23</td>
<td>Mean % change should be within 85 – 115%</td>
</tr>
</tbody>
</table>

**Accuracy and precision of QC samples**

Accuracy and precision of low, medium and high quality control samples (n = 6) were determined by analyzing between intra-day and inter-day. The results of method validation are presented in Table 3.6. The accuracy determined at each concentration level was within limit of ± 15% and at LOQ-QC it was within the limit of ± 20% of the nominal value. The precision determined by calculating %CV value. The % CV for LOQ was within 20%, and for remaining all QC samples, it was within 15%.

**Recovery**

The extraction recovery (Mean ± SD) for eprosartan mesylate and IS was found to be 72.4 ± 4.9% and 66.6 ± 4.1% respectively.
Stability studies: There were no significant changes in concentration and it can be concluded that eprosartan mesylate is stable at different storage conditions. The percentage change was within the limit.