CHAPTER-3

MATERIALS AND METHODS
Verapamil hydrochloride
Gift sample from M/s Dr. Reddy Labs, Hyderabad

Losartan potassium
Gift sample from M/s Dr. Reddy Labs, Hyderabad

Poly(ethylene oxides)  {Polyox WSR 303 & Polyox WSR 301}
Gift sample from Dow Chemicals Asia Pvt., Ltd., Mumbai.

Euragits (Eudragit L 100 & Eudragit S 100)
Gift sample from Degusassa Polymers, Mumbai.

Xanthan gum
Yarrow chemicals, Mumbai.

Guar gum
Yarrow chemicals, Mumbai.

Gum karaya
Yarrow chemicals, Mumbai.

Sodium alginate
Yarrow chemicals, Mumbai.

Ethyl Cellulose
Gift sample from Dow Chemicals Asia Pvt., Ltd., Mumbai.

Microcrystalline cellulose [Aviel PH 102]
Dow Chemicals Asia Pvt., Ltd., Mumbai.

Dicalcium phosphate

Starch 1500

Lactose
Magnesium Stearate

Potassium dihydrogen Phosphate

Sodium hydroxide

Hydrochloric acid

Di sodium hydrogen phosphate

HPLC Grade Acetonitrile
Merck Specialties pvt. Ltd., Mumbai.

HPLC Grade water
Merck Specialties pvt. Ltd., Mumbai.

HPLC Grade Methanol
Merck Specialties pvt. Ltd., Mumbai.

HPLC Grade Acetone
Merck Specialties pvt. Ltd., Mumbai.
EXPERIMENTAL SECTION

3.1: ANALYTICAL METHODS:

3.1.1: Estimation of Verapamil Hydrochloride:

A spectrophotometric method based on the measurement of absorbance at 277 nm in buffer solution, was used in the present study for the estimation of verapamil hydrochloride. (163)

Standard Solution:

100 mg of verapamil hydrochloride was dissolved in 0.1 N HCl in 100 ml volumetric flask and the solution was made up to volume with 0.1N HCl.

Procedure:

The standard solutions of verapamil hydrochloride were subsequently diluted with 0.1N HCl to obtain series of dilutions of containing 10,20,30,40 and 50 µg of verapamil hydrochloride per ml of solution. The absorbances of the above solutions were measured by UV spectrophotometer at 277 nm using 0.1N HCl as blank. The concentrations of verapamil hydrochloride and the corresponding absorbance values are given in table 4.1. The absorbance values were plotted against concentrations of verapamil hydrochloride in graph 4.1 and 4.2. The method obeys Beer’s law in the concentration range of 0-50µg /ml. Reproducibility of method was tested by analyzing six separately weighed samples of verapamil hydrochloride. Thus the method was found to be suitable for the estimation of verapamil hydrochloride in dissolution fluids. Verapamil hydrochloride was also estimated in pH 7.2 phosphate buffer in the similar manner.
3.1.2: Estimation of losartan potassium:

Literature survey reveals that several methods such as thin layer chromatography (164), HPLC and derivative spectrophotometry (165) for the determination of losartan potassium in biological fluids and in dosage forms. In the present work an attempt has been made to develop simple and sensitive spectrophotometric method for the estimation of losartan potassium in bulk and in dosage forms. The method is based on an observation that the solution of losartan potassium exhibits an absorbance maximum at 205nm and obeyed Beer’s law in the concentration range 1-5µg / ml. The statistical analysis of the data indicated a high level of precision for the proposed method. Optical characteristics, regression analysis data and precision of the method are presented in table 4.3.

Standard solution:

100mg of losartan potassium was dissolved in 0.1N HCl in 100ml volumetric flask and the solution was made upto volume with 0.1N HCl.

Procedure:

The standard solution of losartan potassium was subsequently diluted with 0.1N HCl to obtained series of dilutions containing 1,2,3,4 and 5 µg of losartan potassium per ml of solution. The absorbances of the above dilutions were measured in Elico double beam UV spectrophotometer at 205 nm using 0.1N HCl as blank. The concentrations of losartan potassium and the corresponding absorbance values are given in table 4.2. The absorbance values were plotted against concentrations of losartan potassium as shown
in graph 4.3 and 4.4. The method obeyed Beer's law in the concentration range of 0-5\(\mu\)g/ml. Reproducibility of the method was tested by analyzing six separately weighed samples of losartan potassium. Thus the method was found to be suitable for the estimation of losartan potassium contents in dissolution fluids. Losartan Potassium was also estimated in buffer solution of pH 6.8 phosphate buffer in the similar manner.

**3.13: Analytical Method for the Estimation of Verapamil Hydrochloride in the Rabbit Plasma.** (166)

The analytical method used for the estimation of verapamil hydrochloride in the rabbit plasma is RP HPLC. Agilent made HPLC instrument with C 18 Column was used for the analysis. The mobile phase composition used for the estimation of verapamil hydrochloride in the rabbit plasma is a mixture of 0.05 M potassium dihydrogen phosphate: acetonitrile: ophosphoric acid (69.5:30:0.5) (pH 3.6). The flow rate was adjusted to 1 ml/min. The detection was carried out at 278 nm with UV detector.

**3.1.4: Analytical method for the estimation of losartan potassium in the rabbit plasma.** (167)

The analytical method used for the estimation of losartan potassium in the rabbit plasma is RP HPLC. Agilent made HPLC instrument with C 18 Column was used for the analysis. The mobile phase composition used for the estimation of losartan potassium in the rabbit plasma is phosphate buffer solution of dibasic potassium phosphate and disodium hydrogen
phosphate (0.02 M pH 7.0). This buffer was mixed with acetonitrile in the ratio 85:15 (v/v). The flow rate was adjusted to 1 ml/min. The detection was carried out at 250 nm with UV detector.

3.2: Preparation of matrix tablets:

The matrix tablets containing verapamil hydrochloride and losartan potassium were prepared by a direct compression technique. POLYOX WSR 301, WSR 303, Eudragit L 100, Eudragit S 100, ethyl cellulose, sodium alginate, xanthan gum, karaya gum and guar gum were used as polymers for controlling the drug release. The composition of various matrix tablet formulations were given in tables 4.6 to 4.25. The Controlled release tablet formulations consisted of a drug and polymer were prepared in different ratios. The dose of the drug was maintained constantly while the proportion of polymers was varied for various matrix tablets.

3.2.1: Method of Preparation:

The formulations prepared are shown in tables (4.6 to 4.25) together with their compositions. The drug, polymer/s, and diluent were screened through # 40 and preblended using a lab scale double cone blender. The lubricant was added and the blend was mixed again prior to compression. The tablet blends were directly compressed by using a Elite 10 station minipress with 6mm flat round punches. To avoid processing variables all batches of matrix tablets were compressed under identical conditions. All the matrix tablets prepared were further evaluated for physical parameters.
such as weight uniformity, hardness, friability and uniformity of drug content.

3.3: **Evaluation Of Physical Parameters**

3.3.1: **Weight uniformity:**

Twenty tablets from each batch were taken randomly and weighed. The average weight was calculated. Then each tablet was weighed individually and weights of each tablet were noted. The weights of individual tablets were then compared with the average weight. Any deviations in the weight of individual tablets from the calculated average weight were evaluated. This test highly describes that all the tablets of a particular batch should be uniform in weight. If any variations are observed, it should be within the IP limits. The test was considered correct if not more than two tablets fall out side the IP limits out of the twenty tablets evaluated for the test. The weight uniformity values were for different formulations are given in tables 4.26 to 4.31.

Table 3.1
3.3.3: **Hardness Test:**

The hardness of the matrix tablets was determined using a Monsanto tablet hardness tester (Campbell Electronics., Mumbai.) The tablet to be tested was placed in between the fixed and movable jaw after adjusting the reading to zero. By moving the screw knob the force on the tablet was gradually increased until the tablet breaks. The pressure required in kg to break the tablet was noted from the scale on the tester. The hardness of the tablet depends on weight of the material used, space between the upper punch and lower punches at the time of compression and pressure applied during compression. Hardness values were for different formulations are given in tables 4.26 to 4.31.

3.3.4: **Friability Test:**

This test was performed by using Roche Friabilator. This test was performed on twenty tablets from a batch were weighed and placed in the friabilator chamber. The chamber was allowed to rotate 100 revolutions. During each revolution these tablets fall from a distance of six inches to undergo shock. After completion of 100 revolutions, tablets were collected.

### IP LIMITS OF TABLET WEIGHT UNIFORMITY

<table>
<thead>
<tr>
<th>Average Weight</th>
<th>Percentage Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>80 mg or less</td>
<td>10</td>
</tr>
<tr>
<td>More than 80 mg but less than 250 mg</td>
<td>7.5</td>
</tr>
<tr>
<td>250 mg or more</td>
<td>5</td>
</tr>
</tbody>
</table>
from the chamber dedusted and weighed. The loss in weight indicates the friability. The friability values were given in tables 4.26 to 4.31.

3.4: Drug Content Uniformity:

3.4.1: Verapamil Hydrochloride Matrix Tablets:

A matrix tablet of verapamil hydrochloride was taken at random from each batch and crushed to fine powder. The powdered material was transferred into a 100 ml volumetric flask containing 70 ml of distilled water. The flask was shaken occasionally for 30 minutes and the volume was made up to 100 ml mark with distilled water. About 10 ml of the solution was taken and filtered. The filtrate was suitably diluted and the absorbance was measured at 277 nm using UV spectrophotometer (Elico model SL-218). This test was repeated with six tablets from each batch. The amount of verapamil hydrochloride estimated from different batches of tablets was given in the tables 4.26 to 4.28.

3.4.2: Losartan Potassium Matrix Tablets:

Matrix tablet of losartan potassium was taken at random from each batch and crushed to fine powder. The powdered material was transferred into a 100 ml volumetric flask containing 70 ml of distilled water. The flask was shaken occasionally for 30 minutes and the volume was made up to 100 ml mark with distilled water. About 10 ml of the solution was taken and filtered. The filtrate was suitably diluted and the absorbance was measured at 205 nm using UV spectrophotometer (Elico model SL-218). This test was repeated with six tablets from each batch. The amount of losartan
potassium estimated from different batches was given in the tables 4.29 to 4.31.

### 3.4.3: I.R. Spectral Studies

I.R Spectral studies were carried out on some selected matrix tablets by using Bruker Fourier Transfer Infrared Spectrometer. These studies on matrix tablets were performed before they are subjected to dissolution studies to check the drug excipients interactions used in the formulation of matrix tablets for verapamil hydrochloride and losartan potassium. The I.R. spectra of various tablet formulations were shown as figure 4.1 to 4.24.

### 3.5: Drug release studies:

#### 3.5.1: Verapamil Hydrochloride Matrix Tablets:

Dissolution studies on all the matrix tablet formulations were performed as per USP procedure for verapamil hydrochloride extended release tablets in a calibrated eight station dissolution test apparatus (Disso 2000) equipped with paddles employing 900 ml of 0.1 N HCl for first 2 hrs and pH 7.4 phosphate buffer for remaining period of time as dissolution medium. The paddles were operated at 50 rpm and the temperature was maintained at 37±1°C through out the experiment. 5ml of samples were withdrawn at regular intervals up to 24 hours and replaced with equal volume of fresh dissolution medium to maintain a constant volume of dissolution medium through out the experiment. Samples were suitably diluted and drug content was determined by measuring the absorbance at
277 nm using double beam UV spectrophotometer (Elico Model SL-218). Necessary corrections were made for the loss of drug due to each sampling. Cumulative amount of drug released versus square root of time period was plotted to calculate the rate of drug release. The standard deviation of dissolved percent were calculated and represented as error bars in the dissolution figures for six determinations.

The dissolution profiles of all the tablet formulations for verapamil hydrochloride were compared with the marketed SR formulation by using a model independent approach of difference factor, \( f_1 \) and similarity factor, \( f_2 \), with all time points included in the \textit{in vitro} dissolution studies \((168-169)\). The equation for calculating difference factor is

\[
f_1 = \frac{\left[ \sum_{t=1}^{n} | R_t - T_t | \right]}{\left[ \sum_{t=1}^{n} R_t \right]} \times 100
\]

The percent error is zero when the test formulation and reference formulation drug release profiles are identical and increase proportionally with the dissimilarity between the two dissolution profiles. Similarity factor, \( f_2 \) is a logarithmic reciprocal square root transformation of the sum of squared error and is a measurement of the similarity in the percent (%) dissolution between the two curves of marketed and test formulations. The equation for calculating similarity factor is

\[
f_2 = 50 \times \log \left\{ \frac{1}{N} \sum_{J=1}^{N} \left[ 1 + \frac{1}{n} \sum_{J=1}^{N} | R_j - T_j |^2 \right]^{0.5} \times 100 \right\}
\]

Where ‘\( n \)’ is the number of dissolution time and \( R_t \) and \( T_t \) are the reference (theoretical) and test dissolution values at time ‘\( t \)’. Dissolution profile was considered satisfactory if \( f_1 \) values lies below 15 (nearing zero) and \( f_2 \) value
lies more than 50. Two dissolution profiles are considered similar when the $f_2$ value is 50 to 100.

### 3.5.2: Losartan Potassium Matrix Tablets:

Dissolution studies on all the matrix tablet formulations were performed in a calibrated eight station dissolution test apparatus (Disso 2000) equipped with paddles employing 900 ml of 0.1 N HCl for first 2 hrs and pH 6.8 phosphate buffer for remaining period of time as a dissolution medium. The paddles were operated to rotate at 75 rpm and the temperature was maintained at 37±1°C through the experiment. 5ml of samples were withdrawn at regular intervals up to 24 hours and replaced with equal volume of fresh dissolution medium to maintain a constant volume of dissolution medium throughout the experiment. Samples were suitably diluted and drug content was determined by measuring the absorbance at 205 nm using double beam UV spectrophotometer (Elico Model SL-218). Necessary corrections were made for the loss of drug due to each sampling. Cumulative amount of drug released versus square root of time period was plotted to calculate the rate of drug release. The standard deviation of dissolved percent were calculated and represented as error bars in the dissolution figures for six determinations.

### 3.6: Swelling Index

The swelling behavior of a dosage unit was measured by studying its weight gain. The swelling index of selected matrix tablets with natural gums
was determined by placing the tablets in the basket of dissolution apparatus using dissolution medium at 37±0.5°C. After 0.5, 1, 2, 3, 4, 5, 6, 7, 8 and 12 hr’s, each dissolution basket containing tablet was withdrawn and blotted with tissue paper to remove the excess water and weighed on the analytical balance (Shimadzu, Ax 120). The experiment was performed in triplicate for each time point. Swelling index was calculated by using the following formula.

\[
\text{Swelling index} = \frac{\text{(Wet weight of tablet} - \text{Dry weight of tablet})}{\text{Dry Weight of Tablet}}
\]

The swelling index for various selected formulations of matrix tablets were shown in Table No: 4.32.

### 3.7: Pharmacokinetic Studies of Verapamil Hydrochloride

Two optimized controlled release matrix tablets of verapamil hydrochloride based on in vitro dissolution studies were further subjected to in vivo pharmacokinetic studies. Male New Zealand white rabbits weighing 2.0 to 2.5 kg were selected for these studies. The in vivo studies were performed after getting approval from IAEC. These studies were performed by using parallel design. Rabbits were divided into 3 groups, each consisting of 3 animals. The first group received reference standard (verapamil hydrochloride drug solution) and three each were used for selected matrix tablet formulations. Rabbits were selected after checking whether they were used for any other experiments at least 15 days prior to this experimentation. All rabbits were housed in an animal house as per
CPCSEA norms. All the rabbits were fasted overnight on the penultimate day before actual experimentation. They were allowed access to drinking water alone. 18 mg of verapamil hydrochloride (plain drug) dissolved in distilled water was administered orally to the first group of rabbits through oral tube. Few ml of distilled water was pushed through a syringe (without needle) to ascertain that all the verapamil reaches the stomach. The two matrix tablet formulations containing 18mg of verapamil hydrochloride were orally administered to second and third group of rabbits by using a oral tube. After administration, at periodic time intervals of 30 min, 1 hr, 2 hrs,3 hrs, 4 hrs, 5 hrs, 6 hrs, 8 hrs, 12hrs, 16hrs, 20hrs and 24 hrs blood samples were withdrawn using fine gauge needle from the marginal ear vein. Collected blood samples were heparinized and stored in a freezer before analysis. Plasma samples and deproteinizing solution were mixed in a ratio of 4:1 and were added to a 2 ml polypropylene micro centrifuge tube. After capping, the tube contents were vortex mixed for 30 s, and the suspension was centrifuged at 4000 rpm for 10 min. Plasma was separated using micropipette for further quantitative evaluation. The supernatant liquid was collected and diluted with the mobile phase was analyzed by RP HPLC method. The pharmacokinetic parameters such as maximum plasma concentration (C\text{max}), time to reach peak plasma concentration (t\text{max}), t\frac{\text{1}}{2}, AUC (0 to t), Kel and MRT were calculated using software by PK summit solutions and results are given in the table 4.71.
3.8: Pharmacokinetic Studies of Losartan Potassium

Two optimized controlled release matrix tablets of losartan potassium based on in vitro dissolution studies were further subjected to in vivo pharmacokinetic studies. Male New Zealand white rabbits weighing 2.0 to 2.5 kg were selected for these studies. The in vivo studies were performed after getting approval from IAEC. These studies were performed by using parallel design. Three rabbits in each group were utilized in this protocol. Three rabbits were used for reference standard (Losartan potassium drug solution) and three each were used for selected matrix tablet formulations. Rabbits were selected after checking whether they were used for any other experiments at least 15 days prior to this experimentation. All rabbits were housed in an animal house as per CPCSEA norms. All the rabbits were fasted overnight on the penultimate day before actual experimentation. They were allowed access to drinking water alone. 15 mg of losartan potassium (plain drug) dissolved in distilled water was administered orally to the first group of rabbits through oral tube. Few ml of distilled water was pushed through a syringe (without needle) to ascertain that all the losartan reaches the stomach. The two matrix tablet formulations containing 15 mg of losartan potassium were orally administered to second and third group of rabbits by using a oral tube. After administration, at periodic time intervals of 30 min, 1 hr, 2 hrs, 3 hrs, 4 hrs, 5 hrs, 6 hrs, 8 hrs, 12 hrs, 16 hrs, 20 hrs and 24 hrs blood samples were withdrawn using fine gauge needle from the marginal ear vein. Collected blood samples were heparinized and stored in a freezer before analysis. Blood samples were thawed and centrifuged at
14,000 rpm for 15 min. Plasma was separated using micropipette for further quantitative evaluation. Plasma was deproteinated using acetone and the precipitate was separated by centrifugation at 12,000 rpm for 5 min. The supernatant liquid was collected and diluted with the mobile phase was analyzed by RP HPLC method. The pharmacokinetic parameters such as maximum plasma concentration ($C_{\text{max}}$), time to reach peak plasma concentration ($t_{\text{max}}$), $t_{\frac{1}{2}}$, $AUC_{(0 \text{ to } t)}$, $K_e$ and MRT were calculated using software by PK summit solutions and results are given in the table 4.75.

3.9: Stability Studies:

Selected formulations of verapamil hydrochloride and losartan potassium were subjected to accelerated stability studies as per ICH guidelines (170). The matrix tablet formulations such as FVH3, FVH6, FVH9, FVH12, FVH14, FVH16, FVH18, FVH20, FVH24, FVH26, FLP3, FLP6, FLP9, FLP12, FLP14, FLP16, FLP18, FLP20, FLP24 and FLP26 were subjected to accelerated stability studies.

The above said formulations were kept in the HDPE containers after preparation and were stored in thermostated ovens at $40^\circ \text{C} \pm 2^\circ \text{C} / 75% \pm 5% \text{ RH}$ for 6 months. The samples from each formulation were evaluated for the earlier mentioned physical parameters. Further these were subjected to in vitro drug release studies as stated earlier. The results are given in tables 4.80 to 4.89.