Chapter 4
Materials and Methods
The project involves human beings. The whole project was carried out after approval by Independent Ethics committee-**Clinicom**, chaired by Mr. V V Raghavan, “Bhooma”, No. 7, 17th A Cross, Malleshwaram West, Bangalore – 560 055 Karnataka, India (**annexure I**)

4.1. **Demographic Assessment**

The demographic assessment was done in two parts during this study. The first part includes anthropometric analysis of the post menopausal female (PMF) i.e. height, weight and other parameters. The second part includes laboratory profile i.e. hematological profile, biochemical profile, hepatic profile, renal profile.

4.1.1. **Demographic data for anthropometric analysis:**

A prospective data analysis was performed on 500 volunteers who visited the clinic for various medical conditions like irregular bleeding, fatigue, depression and so on. The purpose and procedures of the study were explained to the subjects. All the subjects underwent a physical examination, and those who were taking any chronic medication for any medical ailment or had chronic illness were excluded from this study. The anthropometric data collected includes their name, age, sex, date of birth, height and weight measurement, their food habits like whether they are vegetarian or non vegetarian, tobacco and/or alcohol consumption and the body mass index (BMI) was calculated \[\text{BMI} = \frac{\text{weight (kg)}}{\text{height (m}^2\text{)}}\]

For this purpose the volunteers were handed the patient’s medical questionnaire (**annexure VI**) that was specifically designed for this study. The volunteers were asked to fill the details mentioned in the questionnaire precisely. In case if the volunteer is unable to understand the questions, the accompanying person was requested to fill the questionnaire.

4.1.2. **Demographic data of laboratory parameters:**

In this study the 498 PMF volunteers were divided into six groups according to their age which includes Group I, Group II, Group III, Group IV, Group V and Group VI as per the age of 40 to 45, 46 to 50, 51 to 55, 56 to 60, 61 to 65 and 65 to 70 years respectively.

The laboratory parameters carried out includes

4.1.2.1 Hematological parameters include;

- Hemoglobin value \(\text{Hb}\)
- Red blood corpuscles count \(\text{RBC}\)
⇒ Hematocrit (HCT)
⇒ Total leukocyte count (TLC)
⇒ Platelets
⇒ Mean corpuscular volume (MCV)
⇒ Mean corpuscular hemoglobin (MCH)
⇒ Mean corpuscular hemoglobin concentration (MCHC)
⇒ Differential leukocyte count (DLC) include
  ➢ Neutrophil
  ➢ Lymphocytes
  ➢ Monocytes
  ➢ Eosinophils
  ➢ Basophil

4.1.2.2. Biochemical parameters include;
⇒ Blood glucose
⇒ Serum albumin
⇒ Serum chloride
⇒ Serum potassium
⇒ Serum sodium
⇒ Serum calcium
⇒ Serum urea
⇒ Serum total protein
⇒ Serum C-reactive protein (CRP)

4.1.2.3. Liver function tests (LFTs) include;
⇒ Serum glutamic pyruvic transaminase (SGOT)
⇒ Serum glutamic oxaloacetic transaminase (SGPT)
⇒ Serum bilirubin (total, direct and indirect)
⇒ Serum alkaline phosphatise (ALP)

4.1.2.4. Renal function tests (RFTs) include;
⇒ Serum creatinine
⇒ Blood urea nitrogen (BUN)
4.1.3.  **Procedure for blood and urine collection for laboratory analysis:**

4.1.3.1. **Subject Preparation:**

Before blood collection, it was assured to the subject that venipuncture will be slightly painful but it will be of short duration of time. The subject was made to sit comfortably in a chair extending the arm straight from the shoulder.

4.1.3.1.1. **Blood Collection Procedure:**

The venipuncture site was selected, cleaned with alcohol swab. A tourniquet was tied around the arm just above the area of venipuncture. The needle holder was unscrewed and needle shield was removed. Venipuncture was performed with arm in a downward position by holding the vacutainer with needle. Pushed the vacutainer onto needle. Waited until the first tube is full and blood flow ceases. Removed it from the holder. Place succeeding tubes in holder and pushed onto needle to initiate flow. As soon as blood stops flowing in the last tube, the tourniquet was removed and then needle from the vein. The volume of the blood collected for hematalogical parameter 7mL, biochemical parameters are 2 mL, LFT 2 mL and biochemical parameter 2mL respectively.

4.1.3.1.2. **Procedure for urine sample collection:**

Handed over well labeled urine container to subject for obtaining a urine sample. Instructed the subject to void the first portion of urine. The mid portion of urine should be collected in wide mouth container provided by the laboratory.

4.1.3.2. **Hematological tests using automated hematology analyzer**

The hematological data were analyzed by using automated hematology analyzer (CELL DYN 3500-5 Parts) for the following parameters like: Hb, RBC, HCT, DLC, TLC, MCV, MCH and MCHC.

4.1.3.3. **Biochemical, Hepatic and Renal parameters using auto-analyzer**

The biochemical, LFTs and RFTs were performed by using auto-analyzer (OLYMPUS AU400).
4.2. Method development and validation process for hormone analysis

4.2.1. Method development and validation process for simultaneous estimation of estradiol and estrone in human plasma by LC-MS-MS method.\textsuperscript{24}

A LC-MS-MS chromatographic method using mass detector for the estimation of Estrone and Estradiol in bovine serum albumin has been developed and validated. Sample preparation and analysis has been done. The validation report provides results of standard curve and quality control sample data, recovery, stability data and representative chromatograms.

![Chemical structures of estradiol, estrone, and amlodipine]

**Estrone**

Molecular Formula: \(C_{18}H_{22}O_2\)  
Molecular Weight: 270.37  
Solubility: Acetone

**Estradiol**

Molecular Formula: \(C_{18}H_{24}O_2\)  
Molecular Weight: 272.38  
Solubility: Acetone

**Amlodipine**

Molecular Formula: \(C_{18}H_{21}D_3O_2\)  
Molecular Weight: 275.40  
Solubility: Acetone

*Figure 9: Chemical structure of estradiol, estrone and amlodipine*
The HPLC Alliance HT 2795 series (Waters, USA) is equipped with binary pump, degasser and autosampler with thermostat, thermostated column compartment and control module. The chromatography was on Waters Sun fire C18 column (3.5-μm, 50 mm X 21 mm i.d.) at 30°C temperature. The mobile phase composition was a mixture of 0.1v/v % formic acid buffer and acetonitrile in gradient mode, with a flow rate of 0.25 mL/min.

Mass spectrometric detection was performed on ESI triple quadrupole instrument Quattro Premier (Micromass MS Technologies, Waters, USA) using multiple reaction monitoring (MRM). Data processing was performed on QuanLynx 4.0 software package (Waters).

4.2.1.1. Sample extraction procedure:

A 500-μL volume of standards, controls (bovine serum albumin) and plasma sample was transferred to a 15mL glass test tube. Added 4-mL aliquot of dichloromethane into each tube using multipette plus (eppendorf, USA). The sample was vortex-mixed for 4 min using Multi-Pulse Vortexer (Glas-Col, Terre Haute, USA). The upper aqueous layer was removed and the remaining organic layer was transferred to a 5-mL glass tube and evaporated to dryness using Turbo Vap LV Evaporator (Zymark, Hopkinton, MA, USA) at 50°C under a stream of nitrogen. Then the residue was dissolved in 200-μL of NaHCO₃ (100 mM; pH 10.5) and 200-μL dansyl chloride solution (1mg/mL in acetone) was added followed by vortex for few minutes. The solution was heated for 3 min at 60°C, 25 μL of internal standard (IS) (1.13 μg/mL) was added followed by vortex for few minutes and from the resulting solution 30 μL aliquot was injected into chromatographic system.

4.2.1.2. Bioanalytical method validation

4.2.1.2.1. Calibration and control samples

Working solutions for calibration and quality control samples were prepared from the stock solution by an adequate dilution using water/methanol (1/1v/v). The I.S. working solution (1.13 μg/mL) was prepared by diluting its stock solution with water/methanol (1/1 v/v). Working solutions were added to drug free plasma to obtain the concentration levels of E2 (Estradiol) at 15.64, 31.28, 62.56, 189.58, 631.93, 902.76, 1128.45 and 1253.83 pg/mL. For E1 (Estrone) it was at 16.09, 32.18, 64.37, 195.08, 650.27, 928.96, 1161.20 and 1290.22
pg/mL. Quality control samples were prepared as a bulk, at concentration of 16.04 (lower limit of quantitation, LLOQ), 40.12 pg/mL (low), 501.53 pg/mL (medium) and 1003.07 pg/mL (high) for E2 and concentrations of the quality control samples for E1 were 16.51 pg/mL (lower limit of quantification, LLOQ), 41.28 pg/mL (low), 516.09 pg/mL (medium) and 1032.18 (high).

4.2.1.2.2. Calibration curve

A calibration curve of estradiol and estrone were constructed from a bovine serum albumin processed without an I.S., a zero sample processed with I.S. and eight non-zero samples covering the total range (15-1300 pg/mL), including lower limit of quantification. Eight concentrations were prepared from the stock solution and measured. Linearity was assessed by a weighted (1/x²) least squares regression analysis. The calibration curve had to have a correlation coefficient (r²) of 0.9900 or better. The acceptance criterion for each back-calculated standard concentration was 15% deviation from the nominal value except LLOQ, which was set at 20%.

4.2.1.2.3. Precision and accuracy

The within-batch precision and accuracy was determined by analyzing eight sets of quality control samples in a batch. The between-batch precision and accuracy was determined by analyzing eight sets of seeded quality control samples on three different batches. The quality control samples were randomized daily, processed and analyzed in position either (a) immediately following the standard curve, (b) in the middle of the batch, or (c) at the end of the batch. The acceptance criteria of within-and between-batch precision were 20% or better for LLOQ and 15% or better for the rest of concentrations and the accuracy was 100 ± 20% or better for LLOQ and 100 ± 15% or better for the rest of concentrations.

4.2.1.3. Recovery

Recovery of estradiol and estrone was evaluated by comparing the mean peak areas of the extracted quality control samples to mean peak areas of the reference solution spiked in bovine serum albumin.
4.2.1.4. Stability studies

The bench top stability (at room temperature) of low and high quality control samples were determined by comparing the mean of back-calculated concentrations from the freshly thawed quality control samples with those that were kept on bench top for about 9.0 hrs.

The freeze thaw stability of low and high quality control samples were tested with three freezing periods, where the first storage of 24 hr at below -60°C was followed by two additional periods of 12 to 24 hrs. The percent degradation was determined by comparing the mean of back-calculated concentrations from the three freeze thaw cycles with that of a freshly thawed quality control samples.

Autosampler stability was assessed by storing the low and high quality control samples in auto sampler (10°C) for 25 hrs followed by re-injecting the same samples and comparing the ration of the mean concentrations.

4.2.2. Method development and validation process for estimation of progesterone in human plasma by LC-MS-MS method\textsuperscript{24}.

**Figure 10: Chemical structure of progesterone and Norethisterone**

The HPLC Alliance HT 2795 series (Waters, USA) is equipped with binary pump, degasser and autosampler with thermostat, thermostated column compartment and control module. The chromatography was on Genesis C-18 (length X internal diameter – 50 X 3.6
mm) at 30°C temperature. The mobile phase was HPLC grade Methanol 95% + Ammonium Acetate 5%/v/v, pH 4.5 in gradient mode, with a flow rate of 0.25 mL/min.

Mass spectrometric detection was performed on ESI triple quadrupole instrument Quattro Premier (Micromass MS technologies, Waters, USA) using multiple reaction monitoring (MRM). The main working parameters of the mass spectrometer are summarized in Table 13. Data processing was performed on QuanLynx 4.0 software package (Waters).

4.2.2.1. Extracting procedure:

The required amount of CC/QC sample along with subject sample (0.25mL) from the deep freezer were taken out and allowed them to thaw at room temperature. The thawed samples were vortexed to ensure complete mixing of contents. Added 25µL of I.S. dilution ~ 0.5 µg/mL and vortexed for 10 sec. and added 4.0 mL Tertiary Methyl butyl ether (TBME), again vortexed for 5 minutes followed by centrifuge for 5 minute at 2000 rpm, transferred the organic layer in evaporation test tube and evaporated the solvent under nitrogen flushing at 45°C. Reconstitute the sample with 250 µL of reconstitute solution, transferred into vials and 30 mL of resulting solution was injected into LC-MS-MS.

4.2.2.2. Bioanalytical method validation

4.2.2.2.1. Calibration and control samples

Working solutions for calibration and quality control samples were prepared from the stock solution by an adequate dilution using water/methanol (1/1% v/v). The I.S. working solution (1.50 µg/mL) was prepared by diluting its stock solution with methanol/ammonium acetate (95/5% v/v). Working solutions were added to drug free plasma to obtain the concentration levels of progesterone at 0.11, 0.202, 1.345, 4.076, 13.586, 20.339, 24.504 and 27.227ng/mL. Quality control samples were prepared as a bulk, at concentration of 0.101 ng/mL (lower limit of quantitation, LLOQ), 0.262 ng/mL (low), 13.586 ng/mL (medium) and 24.504 ng/mL (high) for progesterone.

4.2.2.2.2. Calibration curve

A calibration curve of progesterone were constructed from a bovine serum albumin processed without an I.S, a zero sample processed with I.S and eight non-zero samples covering the total range (15-1300 pg/mL), including lower limit of quantification. Eight samples of each concentration were measured. Linearity was assessed by a weighted (1/x²)
least squares regression analysis. The calibration curve had to have a correlation coefficient \((r^2)\) of 0.9900 or better. The acceptance criterion for each back-calculated standard concentration was 15% deviation from the nominal value except LLOQ, which was set at 20%.

4.2.2.2.3. **Precision and accuracy**

The within-batch precision and accuracy was determined by analyzing quality control samples in a batch. The between-batch precision and accuracy was determined by analyzing eight sets of seeded quality control samples on three different batches. The quality control samples were randomized daily, processed and analyzed in position either (a) immediately following the standard curve, (b) in the middle of the batch, or (c) at the end of the batch. The acceptance criteria of within- and between-batch precision were 20% or better for LLOQ and 15% or better for the rest of concentrations and the accuracy was 100 ± 20% or better for LLOQ and 100 ± 15% or better for the rest of concentrations.

4.2.2.3. **Recovery**

Recovery of progesterone was evaluated by comparing the mean peak areas of quality control samples to mean peak areas of reference solution spiked in bovine serum albumin.

4.2.2.4. **Stability studies**

The Bench top stability (at room temperature) of low and high quality control samples were determined by comparing the mean of back-calculated concentrations from the freshly thawed quality control samples with those that were kept on bench top for about 9 hr.

The freeze thaw stability of low and high quality control samples were tested with three freezing periods, where the first storage of 24 hr at below -60°C was followed by two additional periods of 12 to 24 hrs. The percent degradation was determined by comparing the mean of back-calculated concentrations from the three freeze thaw cycles with that of a freshly thawed quality control samples.

Autosampler stability was assessed by storing the low and high quality control samples in auto sampler (10°C) for 25 hrs followed by re-injecting the same samples and comparing the ration of the mean concentrations.
4.3. **Comparison of hormone levels between premenopausal and postmenopausal females**

Comparison of hormone levels (estradiol, estrone and progesterone) were carried out between pre menopausal and post menopausal females of India. Twenty healthy, post menopausal females between the age group of 45 to 60 and twenty healthy pre menopausal females between age group of 18 to 40 years were participated in this part of the study.

The patients were called to the study centre and details about the study procedure were explained which include purpose, nature, advantages, disadvantages, and outcome of the study. The subjects were also explained that the study is approved by the ethics committee. For each women clinical laboratory examination was performed and after confirming their eligibility they were enrolled in the study. (Note: In this part of the study unlike other clinical procedure only the hematology and post menopausal status was confirmed. For hematological test 5 mL of blood sample was collected).

From females who were eligible in the study a 10 mL of blood samples were collected by direct venipuncture. The blood samples were centrifuged immediately at 5000 rpm for 15 minutes. The plasma samples were separated and stored in the deep freezer (-20 °C).

As per the method developed for estrogen and progesterone estimation in plasma the samples were subjected for respective analysis and confirmed.
4.4. **Pharmacokinetic study of hormones in post menopausal females of India**

All the study procedure was carried according to guidelines for bioavailability and bioequivalence studies, Central Drugs Standard Control Organization (CDSCO), New Delhi and guidance for Industry: Bioavailability and Bioequivalence Studies for Orally Administered Drug Products — General Considerations.  

4.4.1. **Pharmacokinetic study of estrogen (estradiol and estrone) tablets in post menopausal females.**

4.4.1.1. **Study Design:**

Open label, single dose, single centre, pharmacokinetic study of estrogen in post menopausal females of India under fasting condition.

4.4.1.2. **Eligibility Assessment:**

The protocol was approved by the Independent Ethics Committee for clinical investigation. For the purpose of the study the following eligibility assessments were carried out before enrolment of any subject into the dosing / sampling phase of the study.

4.4.1.2.1. **Screening:**

The screening was carried out after taking an initial informed consent from all the subjects for study. Screening procedure was performed as per the regulatory guidelines which include;

- Demographic data, including name, age, sex, date of birth, height and weight and BMI
- Medical and treatment history including present complaints (if any), relevant past medical history, family history, history of any allergy to food or drug, medication history in the last three months
- A history of breast cancer.
  - A history of endometrial cancer or other estrogen-dependent neoplasia.
  - A history of endometriosis.
- Undiagnosed vaginal bleeding.
- Active deep vein thrombosis, thromboembolic disorders or a history of these conditions
- A Pap cervical smear to exclude cancer of the cervix and mammography to exclude breast cancer
- Complete physical examination including recording of vital signs (blood pressure, pulse, temperature and respiration) and systemic examination
➢ 12-lead ECG for heart rate, rhythm and specific finding (if any)
  ➢ Chest X-ray (postero-anterior view)
  ➢ Complete blood count
  ➢ *Serum β-HCG at the time of screening for post menopausal female except for those who have undergone bilateral oophorectomy
  ➢ *Serum FSH and serum Estradiol
  ➢ Biochemistry - blood glucose, total protein, serum sodium, potassium, calcium, BUN, serum alkaline phosphatase.
  ➢ Hepatic profile - SGOT, SGPT, Bilirubin (total, direct and indirect)
  ➢ Renal profile - serum creatinine and urea
  ➢ Urine - physical examinations, chemical examination, microscopic examination and *drugs of abuse (Benzodiazepines, opioids and amphetamine, cocaine, THC) *HIV , *HBS Ag and *HCV Ab

Note:
1. * not applicable for the post study laboratory sample that was performed at the end of the study to rule out possible effects to lab parameters
2. **Clinical laboratory tests:**

Prior to start of the study the normal laboratory range for the following tests was obtained from the Clinical laboratory i.e. Intervein labs, Ahmedabad, which is accredited by college of American pathologist (CAP) and National Accreditation Board for Testing and Calibration Laboratories (NABL) laboratory.

The normal range of laboratory parameters is as mentioned below;

*Complete blood count:*

- Hb 12.00-15.00 g %, RBCs 3.80-4.80 cell/mm³, HCT 36.00 - 46.00 %, MCV 83.00 - 101.00 fL, MCH 27.00 - 32.00 pg, MCHC 31.50 - 34.50 %.
- WBCs 4000 - 10000 cells/mm³
- DLC i.e. neutrophil 40.00 - 80.00 %, lymphocyte 20.00 - 40.00 %, monocyte 2.00 - 10.00 %, eosinophil 1.00 - 6.00 %, basophil 0.00 - 2.00 % and platelet 1.50-4.00 cells/mm³.
- Serum β-HCG except for those who have undergone bilateral oophorectomy.

*Biochemical parameters:*

- Blood glucose 60.00 - 140.00 %, serum albumin 3.50 - 5.20 gm/L.
- Serum chloride 98.00 - 107.00 mmol/L, serum potassium 3.50 - 5.10 mmol/L, serum sodium 136.00 - 145.00 mmol/L, serum calcium 8.10 - 10.40 mmol/L, serum urea 17.00 - 49.00 , serum total protein 6.00 - 7.80 gm/L.
- Serum C-reactive protein (CRP).
Chapter 4

Materials and methods

Hepatic profile;
SGOT 8.00 - 33.00 IU/L, SGPT 4.00 - 36.00 IU/L, Bilirubin (direct, indirect and total) 0.3, 0.10 - 1.00, 0.10 - 1.20 µmol/L and Serum Alkaline Phosphatase 30.00 - 120.00 mmol/L.

Renal profile;
Serum creatinine 0.60 - 1.20 µmol/L and blood urea nitrogen (BUN) 8.00 - 23.00 µmol/L.

4.4.1.2.2. Inclusion Criteria:

- Healthy Indian post-menopausal female of any race within the age range of 45 to 65 years
- Willingness to provide informed consent to participate in the study
- Body Mass Index (BMI) > 19.0 Kg/m² and < 29.9 Kg/m² and in the weight range 45 kg to 90 kg.
- Negative serum β-HCG at the time screening
- Postmenopausal status confirmed by:
  - Plasma estradiol concentration < 50 ng/L.
  - Plasma follicle stimulating hormone (FSH) concentration > 30 IU/L.
  - No vaginal bleeding for at least 3 years [if bleeding is difficult to assess due to previous HRT, the Investigator will decide based on clinical history and hormone profile].
- Absence of disease markers of HIV 1 and 2 and hepatitis B and C virus
- Absence of significant disease or clinically significant abnormal laboratory values on the laboratory evaluations, medical history or physical examination during the screening
- Have a normal 12-lead ECG or one with abnormality considered to be clinically insignificant
- Have a normal chest X-ray (P.A. view)
- Comprehension of the nature and purpose of the study and compliance with the requirement of the entire protocol
- Negative Breath alcohol test at the check in of all the periods
- Non-smoker

4.4.1.2.3. Exclusion criteria:

- History / evidence of allergy or hypersensitivity to estrogen or other related drugs
Any major illness in the last three months or any significant ongoing chronic medical illness

- Epileptics
- Renal or liver impairment
- History or current gastro-intestinal diseases influencing drug absorption, except for appendectomy.
- History within 3 years of drug abuse (including cocaine, opiates, amphetamines, etc.)
- History of alcoholism (more than two years), moderate drinkers (more than three drinks per day) or having consumed alcohol within 48 hrs prior to dosing [one drink is equal to one unit of alcohol (one glass wine, half pint beer, one measure of spirit)]
- High caffeine (more than 5 cups of coffee or tea/day) or tobacco (5 or more packets of gutka per day) consumption
- Consumption of grapefruit juice and poppy containing foods within 48 hrs prior to clinic admission
- Participation in any clinical trial within the last three months
- History of difficulty with donating blood or difficulty in accessibility of veins in left or right arm
- Donation of blood (one unit or 350 mL) within three months prior to receiving the first dose of study medication
- Use of any prescription drug therapy within two weeks or over the counter (OTC) drugs or herbal products within one week prior to receiving the first dose of study medication.

4.4.1.3. Dose administration:

Subjects were housed at least 13 hrs before dosing until 48 hrs post dose sample. After overnight fasting (at least 10 hrs) the subjects were administered with one 2 mg estrogen tablet with 240 mL of water.

4.4.1.4. Blood sampling and processing:

The blood samples (1 X 7mL) were collected at: -48.0, -24.0, 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 10.0, 12.0, 16.0, 24.0, 36.0, 48.0, 60.0 and 72.0 hrs in a K3-EDTA vaccutainers. The samples were immediately centrifuged at 5000 (±100) rpm at 5 °C and the plasma was stored at -20 °C until analysis. (The samples -
48.0, -24.0 are ambulatory sample, where the subjects were called to study centre before check in and 60.0 and 72.0 hrs are ambulatory samples, where subjects were called to study centre after check out).

**4.4.1.5. Analysis of estrogens:**

The samples were taken out of the deep freezer and allowed them to thaw at room temperatures. A 500-μL volume of standards, control and plasma samples were transferred to a 15-mL glass test tubes. 4-mL of dichloromethane was added into each tube using multipette plus (eppendorf, USA). The samples were vortex-mixed for 4 min using Multi-Pulse Vortexer (Glas-Col, Terre Haute, USA). The upper aqueous layer was removed and the remaining organic layer was transferred to a 5-mL glass tube and evaporated to dryness using Turbo Vap LV Evaporator (Zymark, Hopkinton, MA, USA) at 50 °C under a stream of nitrogen. Then the residue were dissolved in 200-μL of NaHCO₃ (100 mM; pH 10.5). 200-μL of dansyl chloride solution (1mg/mL in acetone) was added, vortexed and heated for 5 min at 60 °C. 15 μL of I.S. (2.0 μg/mL) was added, vortexed and 25-μL aliquot was injected into chromatographic system.

**4.4.1.6. Pharmacokinetic Analysis:**

The pharmacokinetic parameters like Cmax (maximum serum levels) and tmax (time to reach Cmax) values were determined. Areas under the serum level-time curve (AUC) were calculated according to the trapezoidal rule until the concentration point at time t, and until the last measured concentration point above the lower limit of quantitation at time t-∞. All the parameters were calculated by using validated WinNonlin® 5.3 software.
4.4.2. Pharmacokinetic study of progesterone capsules in post menopausal females.

4.4.2.1. Study Design:

Open label, single dose, single centre, pharmacokinetic study of progesterone in post menopausal females of India under fasting condition.

4.4.2.2. Eligibility Assessment:

The protocol was approved by the Independent Ethics Committee for Clinical investigation. For the purpose of the study the following eligibility assessments were carried out before enrolment of any subject into the dosing / sampling phase of the study:

4.4.2.2.1. Screening:

The screening was carried out after taking an initial informed consent from all the subjects for study. Screening procedure was performed as per the regulatory guidelines which include;

- Demographic data, including name, age, sex, date of birth, height and weight and BMI
- Medical and treatment history including present complaints (if any), family history, history of any allergy to food or drug, medication history in the last three months
- A history of breast cancer.
  - A history of endometrial cancer or other estrogen-dependent neoplasia.
  - A history of endometriosis.
- Undiagnosed vaginal bleeding.
- Active deep vein thrombosis, thromboembolic disorders or a history of these conditions
- A Pap cervical smear to exclude cancer of the cervix and mammography to exclude breast cancer
- Complete physical examination including recording of vital signs (blood pressure, pulse, temperature and respiration) and systemic examination
- 12-lead ECG for heart rate, rhythm and specific finding (if any)
- Chest X-ray (postero-anterior View)
- Complete blood count
- *Serum β-HCG at the time of screening for post menopausal female except for those who have undergone bilateral oophorectomy
- *Serum FSH and serum Estradiol
Chapter 4
Materials and methods

- Biochemical parameters - blood glucose, total protein, serum sodium, potassium, calcium, BUN, serum alkaline phosphatase.
- Hepatic profile - SGOT, SGPT, Bilirubin (total, direct and indirect)
- Renal profile - serum creatinine and urea
- Urine - physical examinations, chemical examination, microscopic examination and *drugs of abuse (Benzodiazepines, opioids and amphetamine, cocaine, THC) *HIV , *HBS Ag and *HCV Ab

Note:
1. * not applicable for the post study laboratory sample that was performed at the end of the study to rule out possible effects on laboratory parameters
2. Clinical laboratory tests:
   Prior to start of the study the normal laboratory range for the following tests was obtained from the Clinical laboratory i.e. Intervein labs, Ahmedabad, which is accredited by college of American pathologist (CAP) and National Accreditation Board for Testing and Calibration Laboratories (NABL) laboratory.

   The normal range of laboratory parameters is as mentioned below;

   *Complete blood count;*  
   Hb- 12.00-15.00 g %, RBCs 3.80-4.80 cell/mm$^3$, HCT 36.00 - 46.00 %, MCV 83.00 - 101.00 fl, MCH 27.00 - 32.00 pg, MCHC 31.50-34.50 %.
   WBCs 4000 - 10000 cells/mm$^3$
   DLC i.e. neutrophil 40.00 - 80.00 %, lymphocyte 20.00 - 40.00 %, monocyte 2.00 - 10.00 %, eosinophil 1.00 - 6.00 %, basophil 0.00 - 2.00 % and platelet 1.50-4.00 cells/mm$^3$.
   Serum β-HCG except for those who have undergone bilateral oophorectomy.

   *Biochemical parameters;*  
   Blood glucose 60.00 - 140.00 %, serum albumin 3.50 - 5.20 gm/L,
   serum chloride 98.00 - 107.00 mmol/L, serum potassium 3.50 - 5.10 mmol/L, serum sodium 136.00 - 145.00 mmol/L, serum calcium 8.10 - 10.40 mmol/L, serum urea 17.00 - 49.00 , serum total protein 6.00 - 7.80 gm/L
   Serum C-reactive protein (CRP).

   *Hepatic profile;*  
   SGOT 8.00 - 33.00 IU/L, SGPT 4.00 - 36.00 IU/L, Bilirubin (direct, indirect and total) 0.3, 0.10 - 1.00, 0.10 - 1.20 µmol/L and Serum Alkaline Phosphotase 30.00 - 120.00 mmol/L.

   *Renal profile;*  
   Serum creatinine 0.60 - 1.20 µmol/L and blood urea nitrogen (BUN) 8.00 - 23.00 µmol/L.)
4.4.2.2.2. Inclusion Criteria:

- Healthy Indian post-menopausal female of any race within the age range of 45 to 65 years
- Willingness to provide informed consent to participate in the study
- Body Mass Index (BMI) ≥ 19.0 Kg/m² and ≤ 29.9 Kg/m² and in the weight range 45 kg to 90 kg.
- Negative serum β-HCG at the screening time.
- Postmenopausal status confirmed by:
  - Plasma estradiol concentration <50 ng/L.
  - Plasma follicle stimulating hormone (FSH) concentration >30 IU/L.
  - No vaginal bleeding for at least 3 years [if bleeding is difficult to assess due to previous HRT, the Investigator will decide based on clinical history and hormone profile].
- Absence of disease markers of HIV 1 and 2 and hepatitis B and C virus
- Absence of significant disease or clinically significant abnormal laboratory values on the laboratory evaluations, medical history or physical examination during the screening
- Have a normal 12-lead ECG or one with abnormality considered to be clinically insignificant
- Have a normal chest X-ray (P.A. view)
- Comprehension of the nature and purpose of the study and compliance with the requirement of the entire protocol
- Negative Breath alcohol test at the check in of all the periods
- Non-smoker

4.4.2.2.3. Exclusion criteria:

- History / evidence of allergy or hypersensitivity to progesterone or other related drugs
- Any major illness in the last three months or any significant ongoing chronic medical illness
- Epileptics
Renal or liver impairment
History or current gastro-intestinal diseases influencing drug absorption, except for appendectomy.
History within 3 years of drug abuse (including cocaine, opiates, amphetamines, etc.)
History of alcoholism (more than two years), moderate drinkers (more than three drinks per day) or having consumed alcohol within 48 hrs prior to dosing [one drink is equal to one unit of alcohol (one glass wine, half pint beer, one measure of spirit)]
High caffeine (more than 5 cups of coffee or tea/day) or tobacco (5 or more packets of gutka per day) consumption
Consumption of grapefruit juice and poppy containing foods within 48 hrs prior to clinic admission
Participation in any clinical trial within the last three months
History of difficulty with donating blood or difficulty in accessibility of veins in left or right arm
Donation of blood (one unit or 350 mL) within three months prior to receiving the first dose of study medication
Use of any prescription drug therapy within two weeks or over the counter (OTC) drugs or herbal products within one week prior to receiving the first dose of study medication.

4.4.2.4. Dose administration:

Subjects were housed at least 13 hrs before dosing until 72 hrs post dose blood sample collection. After overnight fasting (at least 10 hrs) the subjects were administered with one capsule of 200 mg progesterone with 240 mL of water.

4.4.2.5. Blood sampling and Processing:

The blood samples (1 X 7mL) were collected at -72.0, -48.0, -24.0, 0.0, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, 7.0, 8.0, 10.0, 12.0, 16.0, 24.0, 32.0, 36.0 and 48.0 hrs in K3-EDTA vacutainer. Then the samples were centrifuged at 5000 rpm (±100) for 5 minutes at 5 °C (±1) and the separated plasma was stored in the deep freezer (-20 °C) till the analysis. (The samples -72.0, -48.0 hrs are ambulatory samples, where subjects were called to study centre before check in).
4.4.2.6. Analysis of Progesterone:

The progesterone was analyzed by validated LC-MS-MS method. These were taken out of the deep freezer and allowed to thaw at room temperature. A 0.25 mL of standard, control and plasma samples were vortexed to ensure complete mixing of contents. I.S. dilution ~0.5 µg/mL (25 µL) was added, vortexed for 10 sec. and 4.0 mL TBME was added, vortexed for 5 minutes and centrifuge for 5 minute at 2000 rpm. After that the organic layer was transferred to evaporation test tube and was evaporated the solvent under nitrogen flushing at 45 ºC. The samples were reconstituted with 250 µL of reconstitute solution, transferred into vials and 30 µL of the resulting solution was injected into LC-MS-MS.

4.4.2.7. Evaluation of pharmacokinetic parameters:

Pharmacokinetic parameters like Cmax (maximum serum levels) and tmax (time to reach Cmax) values were determined. Areas under the serum level-time curve (AUC) were calculated according to the trapezoidal rule until the concentration point at time t, and until the last measured concentration point above the lower limit of quantitation at time t-∞. All the parameters were calculated by using validated WinNonlin® 5.3 software.