

## **CHAPTER – 3**

### **ANALYTICAL METHODS**

In this investigation, liposomes of LN and LEU were prepared and prepared liposomes were further formulated as nasal and DPI formulations for contraception. The chemical characterization of liposomes and developed nasal and DPI formulations were also carried out to determine the drug entrapment efficiency, PC and CHOL content. The stability studies were conducted to determine the percent drug retained in liposomes and liposomal formulations over storage of 6 months period. *In vitro* drug diffusion studies followed by *in vivo* studies in rats were also carried out. The analytical methods employed in these investigations are discussed below.

### 3.1 ESTIMATION OF PC/HSPC

The spectrophotometric method developed by Stewart and Charles, 1980, was used for estimation of PC wherein phospholipid forms a blood red colored complex with ammonium ferrothiocyanate in organic solution.

#### 3.1.1 REAGENTS

##### Ammonium ferrothiocyanate solution (0.1 M)

27.03 gm of ferric chloride hexahydrate and 30.4 gm of ammonium thiocyanate were dissolved in distilled water and volume was made up to 1000 ml. The solution was stored in an amber colored bottle at room temperature.

##### Stock solution of PC/HSPC

A 100 µg/ml solution of PC/HSPC was prepared in chloroform.

#### 3.1.2 METHOD

Suitable aliquots of the stock solution of EPC were transferred to calibrated centrifuge tubes. The volume in this tube was made up to 3 ml with chloroform. To each tube was added, 2 ml of the Ammonium ferrothiocyanate solution was added and the contents vortexed on a cyclomixer for 2 min. The contents were then centrifuged at 2750 rpm for 5 min to separate the chloroform layer. The chloroform layer was removed using a glass syringe with a long needle. The absorption maxima ( $\lambda_{max}$ ) was determined by scanning 300 µg/3ml solution against the reagent blank on UV-Visible

Spectrophotometer. The absorption of all the prepared solutions was then measured at the absorption maxima, 485 nm, against the reagent blank. The readings were recorded in triplicate and the experiment was repeated on 3 consecutive days using freshly prepared stock solutions each time. Mean values ( $n=9$ ) along with the standard error mean are recorded in Table 3.1 and the regressed calibration curve is shown in Figure 3.1.

## 3.2 SPECTROPHOTOMETRIC DETERMINATION OF CHOLESTEROL

Zlatkis, Zak and Boyle's method (cross ref. Goel, 1988) was used wherein CHOL in acetic acid forms a complex with ferric chloride and sulfuric acid.

### 3.2.1 REAGENTS

- (i) Stock solution of CHOL: A 1 mg/ml solution of cholesterol was prepared in glacial acetic acid.
- (ii) Ferric chloride solution: A 0.05% w/v solution of Ferric chloride, ( $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ), was prepared in glacial acetic acid (aldehyde free) and stored at room temperature in a glass bottle.

Table 3.1 Calibration curve for the estimation of Phosphatidyl choline

Concentration (µg/3ml)	Absorbance (±SEM)
0	0
100	0.150 (0.002)
200	0.296 (0.003)
300	0.443 (0.001)
400	0.586 (0.003)
500	0.734 (0.003)
600	0.906 (0.002)
700	0.998 (0.005)

\*n=9

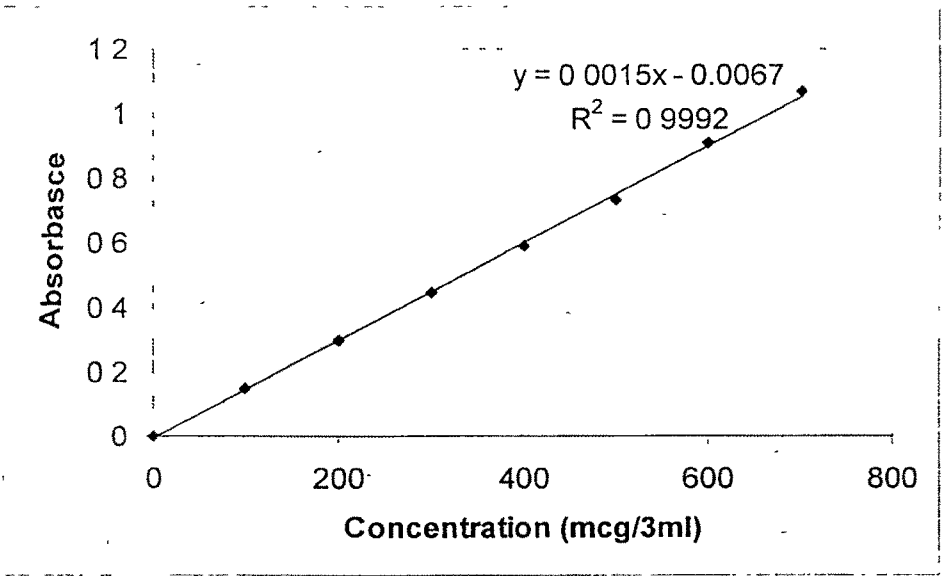


Figure 3.1. Regressed calibration curve for PC/HSPC

### 3.2.2 METHOD

Suitable aliquots of the stock solution of CHOL were transferred to 10 ml volumetric flasks. 4 ml of ferric chloride solution and 4 ml sulfuric acid were added. The solutions were made up to volume with glacial acetic acid, stoppered tightly and mixed by repeated inversion and were allowed to stand for 20 to 30 min. The absorbance of solutions was read against reagent blank on UV-Visible Spectrophotometer. The absorption maxima ( $\lambda_{\max}$ ) was determined by scanning 100  $\mu\text{g/ml}$  solution against the reagent blank. The absorption of all the prepared solutions was then measured at the absorption maxima, 485 nm, against the reagent blank. The readings were recorded in triplicate and the experiment was repeated on 3 consecutive days using freshly prepared stock solutions each time. Mean values ( $n=9$ ) along with the standard error mean are recorded in Table 3.2 and the regressed calibration curve is shown in Figure 3.2.

### 3.3 ESTIMATION OF PC AND CHOL IN LIPOSOMES

The liposomes after separation from the free drug were suspended in to 1 ml water. PC and CHOL were estimated in liposomes by the procedure given below.

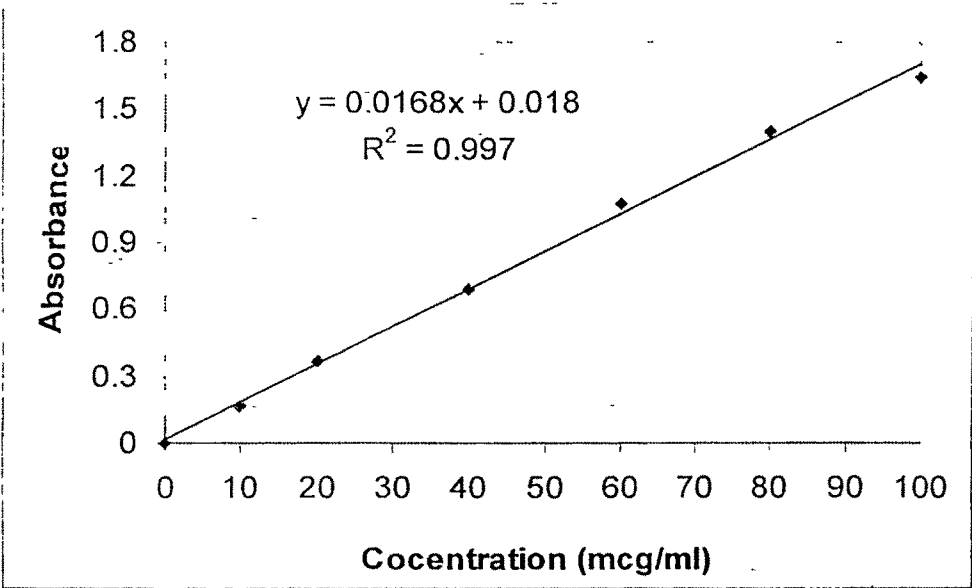
Accurately measured 0.2 ml of liposomal suspension was transferred to a calibrated centrifuge tube and the volume was made up to 2 ml with water. 2 ml of chloroform was added to the content of the centrifuge tube followed by vortexing for 30 seconds and then centrifuging at 2750 rpm for 10 minutes. The lower chloroform layer was separated using a glass syringe with a long needle and transferred into a 10 ml volumetric flask after passing it through a bed of anhydrous sodium sulphate. The extraction procedure was repeated twice with 2 ml portions of chloroform and the volume of the combined extract was made up to 10 ml with chloroform. 3 ml of this chloroform extract was transferred into 10 ml volumetric flask and analyzed for its PC content using the method described in this Chapter (Section 3.3).

Accurately measured 2 ml of the remaining chloroform that contains CHOL was transferred to 10 ml volumetric flask and the solvent was evaporated to dryness. The contents of the flask were then analyzed for its CHOL content using the method described in this Chapter (Section 3.4).

**Table 3.2 Calibration curve for the estimation of CHOL**

Concentration (µg/ml)	Absorbance* (±SEM)
10	0.166 (0.002)
20	0.347 (0.003)
40	0.693 (0.002)
60	1.072 (0.004)
80	1.398 (0.003)
100	1.644(0.003)

\*n=9



**Figure 3.2. Regressed calibration curve for CHOL**

### 3.4 LEVONORGESTREL

#### 3.4.1 SPECTROPHOTOMETRIC DETERMINATION OF LN

The spectrophotometric method used for estimation of LN is based on the principle that LN with INH solution gives yellow color as described in USP XXI

##### 3.4.11 Reagents

- (i) INH Solution Prepared as described in British pharmacopoeia
- (ii) Stock solution of LN A 100 µg/ml solution of LN was prepared in INH solution

##### 3.4.12 Method

Appropriate aliquots of the stock solution of the LN were transferred to 10 ml volumetric flasks and were diluted to 10 ml with INH reagent. The absorption maxima ( $\lambda_{max}$ ) was determined by scanning 10 µg/ml solution against the reagent blank on UV-Visible Spectrophotometer. The absorption of all the prepared solutions was then measured at the absorption maxima, 339 nm against the reagent blank. The readings were recorded in triplicate and the experiment was repeated on three consecutive days using freshly prepared stock solutions each time. Mean values (n=9) along with the standard error mean are recorded in Table 3.3. The regressed values of absorption were plotted graphically against the concentration, as shown in Figure 3.3.

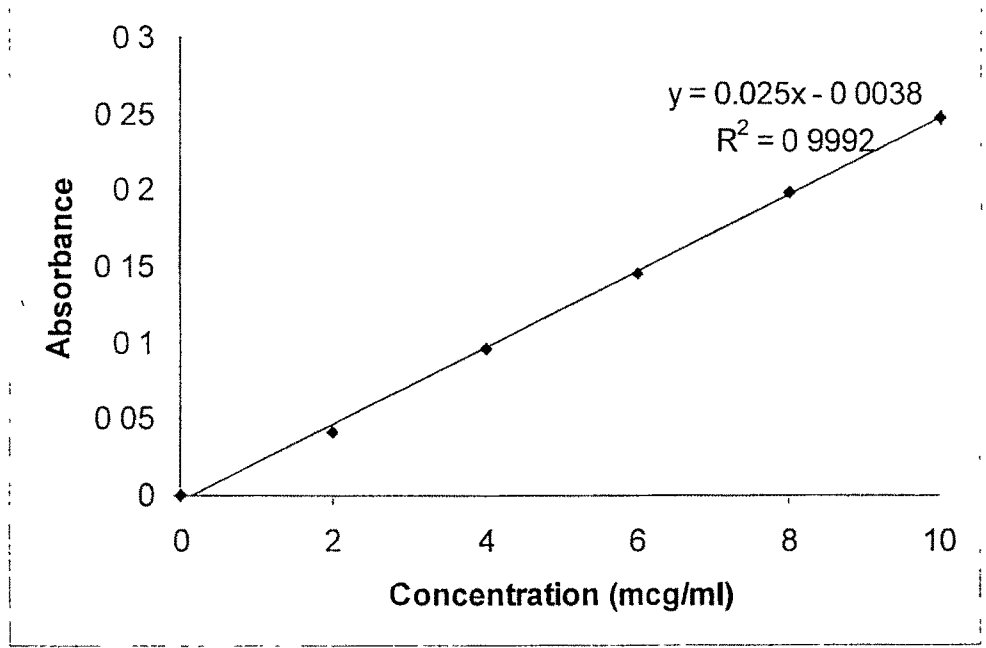
##### Interference of formulation components

The interference of PC, CHOL, CS, CP and sugars (sucrose and lactose) in the estimation of LN was determined by measuring the absorption of the maximum used concentration of these formulation components at 339 nm against the reagent blank. The observations are recorded in Table 3.7.

**Table 3.3 Calibration curve for the estimation of Levonorgestrel by spectrophotometry**

Concentration (µg/ml)	Absorbance* (±SEM)
2	0.042 (0.001)
4	0.096 (0.003)
6	0.145 (0.002)
8	0.198(0.002)
10	0.247(0.003)

\*n=9



**Fig 3.3 Regressed calibration curve for the estimation of LN by spectrophotometry**



Table 3.4 Calibration curve for the estimation of Levonorgestrel by Spectrofluorimetry

Concentration (pg/ml)	Fluorescence intensity * ( $\pm$ SEM)
200	0.027 (0.001)
400	0.061 (0.003)
600	0.101 (0.002)
800	0.142 (0.002)
1000	0.179 (0.003)

\*n=9

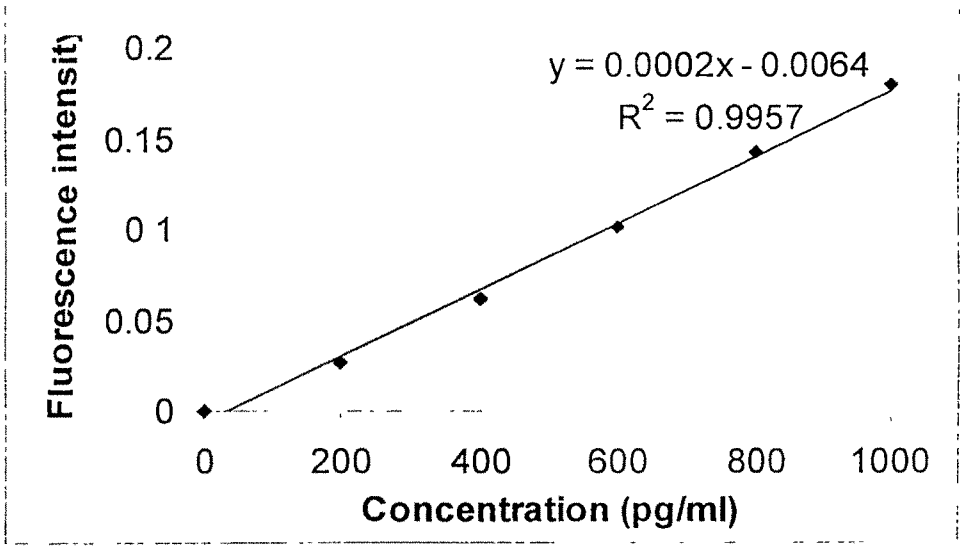
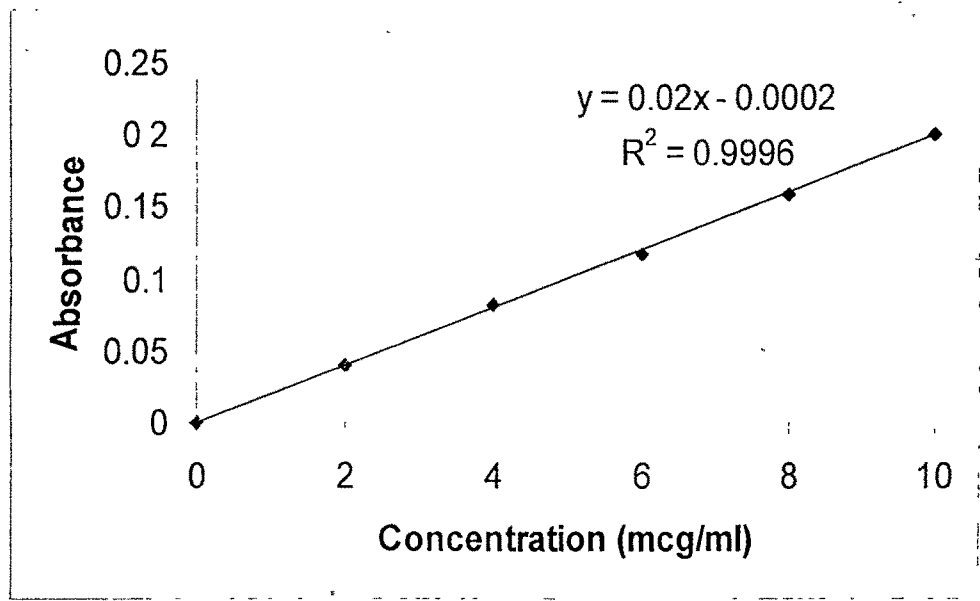


Figure 3.3 Regressed calibration curve for the estimation of LN by spectrofluorimetry-

**Table 3.5 Calibration curve for the estimation of Leuprolide acetate by spectrophotometry**

Concentration (µg/ml)	Absorbance* (±SEM)
2	0.04 (0.001)
4	0.081 (0.002)
6	0.117 (0.002)
8	0.159 (0.003)
10	0.201 (0.002)

\*n=9

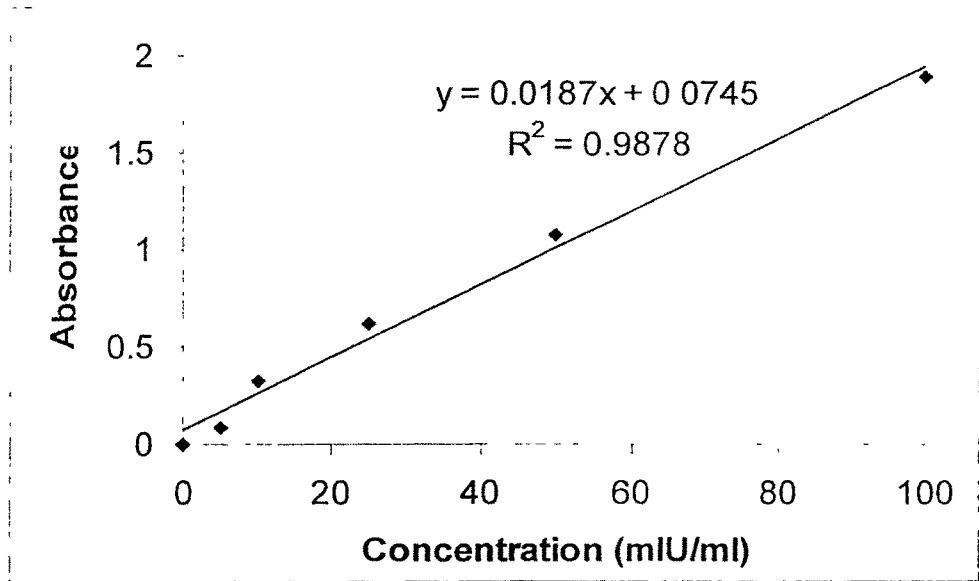


**Figure 3.5. Regressed calibration curve for the estimation of LEU by spectrophotometry**

**Table 3.6 Calibration curve for the estimation of Leuprolide acetate by Radioimmunoassay**

Concentration (mIU/ml)	Absorbance* (±SEM)
0	0
5	0.084 (0.001)
10	0.33 (0.003)
25	0.62 (0.002)
50	1.08 (0.004)
100	1.89 (0.003)

\*n=9



**Figure 3.6. Regressed calibration curve for the estimation of LH by RIA**

### **3.4.2 SPECTROFLUORIMETRIC METHOD FOR ESTIMATION OF LN IN BLOOD.**

The spectrofluorimetric method as described by Andrew and Leo, 1975, was used for estimation of LN concentration in plasma, by measuring sulfuric acid-induced fluorescence at an emission  $\lambda_{\text{max}}$  of 520nm with an excitation  $\lambda_{\text{max}}$  of 460 nm

#### **3.4.21 Reagents**

80% Sulphuric acid in ethanol In 1000 ml volumetric flask, 200ml of ethanol was transferred 800ml of sulphuric acid was added slowly to the flask Volume up to the mark was adjusted with ethanol (loss occurred due to the heat generation)

Stock solution of LN. Stock solution of LN. A 1 $\mu$ g/ml solution of LN prepared in ethanol

10ml of fresh blood was collected from a single rat into heparin coated vials and plasma was separated using centrifugation at 6,000 rpm

#### **3.4.22 Method**

Appropriate aliquots of the stock solution of the LN were transferred to test tubes and diluted to 1 ml with fresh plasma The resulting solutions were kept in Freeze for 30 min The solutions were then extracted with 2 ml and 1 ml methylene chloride two times. The methylene chloride samples were combined and again extracted with 2 ml 80% sulphuric acid in ethanol The sulphuric acid layer was then removed using a glass syringe with a long needle The relative fluorescence intensity of the solutions were measured at excitation wavelength of 460nm and emission wavelength of 520nm determined in 1cm cell against reagent blank The readings were recorded in triplicate and the experiment was repeated on three consecutive days using freshly prepared stock solutions each time Mean values (n=9) along with the standard error mean are recorded in Table 3.4 The regressed values of absorption were plotted graphically against the concentration, as shown in Figure 3 4.

### **3.4.3 ESTIMATION OF LN ENTRAPPED IN LIPOSOMES**

Accurately measured 0.1 ml of liposomal suspension was transferred to 5 ml volumetric flask and volume was made up to 5 ml with INH reagent. The resulting solution was diluted suitably if necessary and the LN content was analyzed by measuring the absorbance at  $\lambda_{\max}$  379 nm against the reagent blank as described in this Chapter (Section 3.4.1).

### **3.4.4 ESTIMATION OF UNENTRAPPED LN**

The sedimented free drug after separation from liposomes was diluted to 1 ml with hydrating medium and mixed well. 0.1 ml of the resulting suspension was transferred to clean and dry, separate, 5 ml volumetric flask and volume was made up to 5 ml with INH reagent. The resulting solution was further diluted with INH reagent if necessary and the LN content was analyzed by measuring the absorbance at  $\lambda_{\max}$  379 nm against the reagent blank as described in this Chapter (Section 3.4.1).

### **3.4.5 ESTIMATION OF LN IN CARBOPOL (CP) AND CHITOSAN (CS) SUSPENSIONS:**

0.2 ml of the CP or CS suspension was diluted to 1 ml with water and resulting suspension was centrifuged at 3,000 rpm for 15 minutes. Supernatant was decanted and the sediment after sufficient washing was dissolved in 5 ml of INH reagent. The drug content was then analyzed by the method described in this Chapter (Section 3.4.1).

### **3.4.6 ESTIMATION OF LEVONORGESTREL IN DRY POWDER INHALER FORMULATIONS:**

100 mg of the powder was rehydrated with 1 ml distilled water with gentle, occasional agitation. The rehydrated liposomal dispersion was separated from the leaked drug by centrifugation at 3,000 rpm for 15 min and analyzed for PDE using method described in this Chapter (Section 3.4.1).

### **3.4.7 ESTIMATION OF LEVONORGESTREL RETENTION IN LIPOSOMES DURING THE DRUG RETENTION STUDIES OF PREPARED**

#### **FORMULATIONS:**

In case of liposomes, and liposomal formulation with CS and CP, 0.2 ml of liposomal suspension, stored at each temperature ( $2-8^{\circ}\text{C}$  and  $25\pm 2^{\circ}\text{C}$ ) were withdrawn at a specific time intervals during six months drug retention studies. After 2 times dilution with water, liposomal suspensions were subjected to centrifugation at 3,000 rpm for 15 minutes to separate untrapped drug. The purified liposomal suspension was analyzed by measuring the absorbance at  $\lambda_{\text{max}}$  379 nm against the reagent blank as described in this Chapter (Section 3.4.3).

In case of LDPI formulations, 100 mg of samples stored at each temperature ( $2-8^{\circ}\text{C}$ ,  $25\pm 2^{\circ}\text{C}$  and  $40\pm 2^{\circ}\text{C}$ ) were withdrawn at a specific time intervals during drug retention study and dispersed in 1 ml of water. The remaining procedure was carried out as in case of liposomal suspension.

### **3.4.8 ESTIMATION OF LEVONORGESTREL IN THE DIFFUSION MEDIUM:**

0.5 ml of the sample (drug in diffusion medium) withdrawn at predefined time intervals was transferred to a clean and dry volumetric flask and volume was made up to 10 ml with INH reagent. The resulting solution was further diluted if necessary and the LN content was analyzed by measuring the absorbance at  $\lambda_{\text{max}}$  379 nm against the reagent blank as described in this Chapter (Section 3.4.1).

### **3.4.9 ESTIMATION OF LEVONORGESTREL IN PLASMA:**

200  $\mu\text{l}$  of blood samples were collected at different time points as described in Chapter 5 and 6 and plasma were separated using centrifugation at 6,000 rpm. The plasma samples were further treated as described earlier in this Chapter (Section 3.4.2).

**Table 3.7: Interference of excipients used in the formulations**

Excipients	Absorbance at 339 nm	Absorbance at 240 nm	Peak area
PC	0.001	0.002	0.00
CHOL	0.00	0.00	0.00
Chitosan	0.002	0.002	0.00
Carbopol	0.001	--	0.00
Lactose (Sorbolac/Pharmatose)	0.00	0.00	0.00
Sucrose	0.00	0.00	0.00

### 3.5 LEUPROLIDE ACETATE

#### 3.5.1 SPECTROSCOPIC ESTIMATION OF LEUPROLIDE ACETATE

The spectroscopic determination of LEU is based on the fact that UV spectra of LEU gave absorption maxima at 240 nm in 0.1 N NaOH (Akwete and Hsu, 1993)

##### 3.5.11 Reagents

(i) 0.1 N NaOH solution

4 gm Sodium Hydroxide dissolved in 1000 ml of double distilled water

(ii) Stock solution of LEU

100 µg/ml solution of LEU was prepared in 0.1 N NaOH solution

##### 3.5.12 Method:

Appropriate aliquots of the stock solution of LEU were transferred to 10 ml volumetric flasks and were diluted to 10 ml with 0.1 N NaOH solution. The absorption maxima ( $\lambda_{max}$ ) was determined by scanning 10 µg/ml solution against the reagent blank on UV-Visible Spectrophotometer. The absorption of all the prepared solutions was then measured at the absorption maxima, 240 nm against the reagent blank. The readings were recorded in triplicate and the experiment was repeated on three consecutive days using freshly prepared stock solutions each time. Mean values (n=9) along with the standard error mean are recorded in Table 3.5. The regressed values of absorption were plotted graphically against the concentration, as shown in Figure 3.5

##### Interference of formulation components:

The interference of PC, CHOL, CS, phosphate buffer and sugars (sucrose and lactose) in the estimation of LEU was determined by measuring the absorption of the maximum used concentration of these formulation components at 240 nm against the reagent blank. The observations are recorded in Table 3.7



### **3.5.2 PREPARATION OF CALIBRATION CURVE FOR THE ESTIMATION OF LH BY RADIOIMMUNOASSAY:**

Plasma LH concentration was measured by radioimmunoassay method using Monobind ELISA kit

#### **3.5.21 Reagents**

##### (i) LH Calibrators

Six (6) vials of references for LH antigen at levels of 0, 5, 10, 25, 50 and 100 mIU/ml

##### (ii) Enzyme-LH antibody conjugate and Biotinylated monoclonal antibody

Enzyme labeled antibody, biotinylated monoclonal mouse IgG in buffer, dye and preservative

##### (iii) Streptavidin coated tubes

##### (iv) Wash solution

A surfactant in Tris buffered saline

##### (v) Substrate A

Tetramethylbenzidine in buffer

##### (vi) Substrate B

Hydrogen peroxide in buffer

##### (vii) Stop solution

1N HCl

#### **3.5.22 Method**

The following steps were carried out:

- (i) To the streptavidin coated tubes, 50 $\mu$ l control samples (LH Calibrators) were added
- (ii) 100  $\mu$ l of the biotinylated/enzyme labeled antibodies to each tube was added to the bottom of the coated tubes
- (iii) Tube rack was swirled gently for 30 seconds and incubated for 60 min at room temperature
- (iv) The tube contents were decanted completely. These tubes were washed two times with 4 ml of wash buffer
- (v) 200  $\mu$ l of working substrate solution was added to all tubes and incubated at room temperature for 20 minutes
- (vi) 2 ml of stop solution was added to each tube to stop the reaction and LEU content were estimated in each tube using Spectrophotometer at the absorption maxima, 450 nm against the reagent blank. The readings were recorded in triplicate and the experiment was repeated on three consecutive days using freshly prepared stock solutions each time. Mean values (n=9) along with the standard error mean are recorded in Table 3.6. The regressed values of absorption were plotted graphically against the concentration, as shown in Figure 3.6

### **3.5.3 ESTIMATION OF LEU ENTRAPPED IN LIPOSOMES:**

Accurately measured 0.2 ml of liposomal suspension of LEU was transferred to clean and dry, 5 ml volumetric flask. To it was added 1ml 10% triton X-100 in water and warmed at 60°C till the solution became clear and the volume was made up to 5 ml with 0.1 N NaOH solution. The resulting solution was further diluted if necessary and LEU content was analyzed using spectroscopic method by measuring the absorbance at  $\lambda_{max}$  240 nm against the reagent blank as described in this Chapter (Section 3.5.1)

### **3.5.4 ESTIMATION OF UNENTRAPPED LEU IN SUPERNATANT:**

Accurately measured 0.2 ml of the supernatant liquid was transferred to clean and dry, separate, 5 ml volumetric flask and volume was made upto 5 ml with 0.1N NaOH.

The resulting solution was further diluted if necessary and the LEU content was analyzed using spectrophotometric method by measuring the absorbance at  $\lambda_{\max}$  240 nm against the reagent blank as described in this Chapter (Section 3.5.1)

### **3.5.5 ESTIMATION OF LEU IN THE PREPARED CHITOSAN**

#### **SUSPENSION:**

0.2 ml of the CS solution containing drug was taken and diluted to 5 ml with 0.1N NaOH. The resulting solution was further diluted if necessary and the LEU content was analyzed against the reagent blank prepared in similar way by the method described in this Chapter (Section 3.5.1)

### **3.5.6 ESTIMATION OF LEU IN DRY POWDER INHALER**

#### **FORMULATIONS:**

A fraction of the powder was rehydrated with Phosphate buffer pH 5.2 with gentle, occasional agitation. The rehydrated liposomal dispersion was separated from the leaked drug by centrifugation at 15,000 rpm for 30 min and analyzed for PDE using method described in this Chapter (Section 3.5.1)

### **3.5.7 ESTIMATION OF LEUPROLIDE ACETATE RETENTION IN LIPOSOMES DURING THE DRUG RETENTION STUDIES OF PREPARED FORMULATIONS:**

In case of liposomes, and liposomal formulation with CS, 0.2 ml of liposomal suspension, stored at each temperature (2-8°C and 25±2°C) were withdrawn at a specific time intervals during six months drug retention studies and after 2 times dilution with diffusion medium subjected to centrifugation at 15,000 rpm for 30 minutes to separate untrapped drug. The purified liposomal suspension was analyzed by measuring the absorbance at  $\lambda_{\max}$  240 nm against the reagent blank as described in this Chapter (Section 3.5.3)

In case of LDPI formulations, 100 mg of samples stored at each temperature (2-8°C, 25±2°C and 40±2°C) were withdrawn at a specific time intervals during stability study and dispersed in 1 ml diffusion medium. The remaining procedure was carried out as in case of liposomal suspension (Section 3.5.3)

### **3.5.8 ESTIMATION OF LEUPROLIDE ACETATE IN THE DIFFUSION MEDIUM:**

0.5 ml of the sample (drug in diffusion medium) withdrawn at predefined time intervals was transferred to a clean and dry volumetric flask and volume was made up to 5 ml with 0.1N NaOH solution. The resulting solution was further diluted if necessary and the LEU content was analyzed by measuring the absorbance at  $\lambda_{\text{max}}$  240 nm against the reagent blank as described in this Chapter (Section 3.5.1).

### **3.5.9 ESTIMATION OF LEU IN SERUM:**

During in vivo studies, at different time points, blood samples were collected and allowed to stand for some time to separate the serum and serum samples were analyzed by radioimmunoassay as described in this Chapter (Section 3.5.2).

## **3.6 RESULTS AND DISCUSSION**

PC and CHOL content of liposomes were determined by suitable techniques. PC was determined using Stewart assay. The absorption maxima, determined by scanning a solution of concentration 300 µg/3ml on Spectrophotometer, was found to be 485 nm. The absorbance of all the prepared solutions were then measured at the absorbance maxima. The results are recorded in Table 3.1 and shown graphically in Figure 3.1. Regression analysis of the data proved the linearity of the data ( $R^2 = 0.9992$ ) in the concentration range of 100-700 µg/3ml. The absorption of the solution was found to increase during first 5 minutes of preparation. Hence, all the readings were taken 5 minutes after the completion of color development.

Zlatkis, Zak and Boyle's method was used for the estimation of CHOL. The absorbance maxima determined by scanning solution of concentration 20 µg/ml on Spectrophotometer was found to be 550 nm. The absorbance of all the prepared solutions were then measured at the absorbance maxima. The results are recorded in Table 3.2 and shown graphically in Figure 3.2. Regression analysis of the data proved the linearity of the data ( $R^2 = 0.997$ ) in the concentration range of 10-100 µg/ml.

LN in liposomes was estimated by direct addition of the drug entrapped liposomes in to reagent blank as the liposomal constituents were soluble in the INH reagent. The

absorption maxima, determined by scanning a solution of 10 µg/ml on Spectrophotometer was found to be 339 nm. The absorbance of all the prepared solutions was then measured at the absorbance maxima. The results are recorded in Table 3.3 and shown graphically in Figure 3.3. Regression analysis shows that the curve is linear in the concentration range of 0-10 µg/ml ( $R^2 = 0.9992$ ). The same method could be successfully used for the determination of LN during characterization procedures, stability studies and in vitro diffusion studies. For the determination of LN in plasma spectrofluorimetry was used. The relative fluorescence intensity of all the prepared solutions was then measured at the excitation wavelength. The results are recorded in Table 3.4 and shown graphically in Figure 3.4. Regression analysis shows that the curve is linear in the concentration range of 200-1000 pg/ml ( $R^2 = 0.995$ ).

LEU in liposomes was estimated by breaking the liposomes using 10% triton X-100 in water, making up the volume with 0.1 N NaOH by spectroscopic method. Calibration curve was plotted between concentration 0-10 µg/ml. The results are recorded in Table 3.5 and shown graphically in Figure 3.5. Regression analysis of the data show that the curve is linear in the concentration range of 0-10 µg/ml ( $R^2 = 0.999$ ). The same method was successfully used for the determination of LEU during characterization procedures, stability studies and in vitro diffusion studies. Determination of LEU in plasma was carried out by radioimmunoassay using commercial kit (Monobind ELISA). Calibration curve was plotted between concentration 0-100 mIU/ml. The results are recorded in Table 3.6 and shown graphically in Figure 3.6. Regression analysis of the data show that the curve is linear in the concentration range of 0-100 mIU/ml ( $R^2 = 0.994$ ).

The interference of the formulation components, PC, CHOL, sugars, chitosan and carbopol was ascertained by analyzing the maximum used concentrations at corresponding wavelength of the drug. The results shown in Table 3.7 indicate no significant differences ( $p > 0.05$ ) in the absorbance values/peak area of formulation components in the used concentrations.

### 3.7 REFERENCES

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