CHAPTER 3

ANALYTICAL METHODS

3.1 INTRODUCTION

The analytical methods used in studies of drug-containing liposomes should, in addition to possessing the desired characteristics of accuracy, precision, reproducibility, ruggedness, robustness etc., also possess the ability to be used in conjunction with techniques common to liposomal studies. These techniques, which ensure minimum interference from the other components of the formulation, include methods like Bligh-Dyer two-phase extraction, dialysis, Sep-Pak minicolumn extraction or ultrasonic disruption (New, 1990). The methods used should, preferably, be stability indicating, which would, when used, draw attention to any potential incompatibility between the various components of the liposomes.

3.2 EXPERIMENTAL

3.2.1 DRUGS

Cyclosporine (U.S.P.XXIV) was received as a gift sample from R.P.G. Life Sciences, Ankleshwar. Leuprolide acetate was received as a gift sample from Takeda chemical industries, Osaka, Japan. Calf thymus DNA was a gift from Biotechnology division, M.S.University of Baroda.

3.2.2 REAGENTS

Hydrogenated Soya Phosphatidyl choline was purchased from Lipoid., Germany.; Cholesterol, ferric chloride hexahydrate, ammonium thiocyanate, sodium nitrate, picric acid, sodium chloride, anhydrous sodium sulphate, glacial acetic acid (aldehyde free), chloroform, Tetrahydrofuran, methanol, Tris hydrochloride, Ethylene diamine tetra acetic acid and sodium hydroxide (analytical reagent grade) were purchased from S.D.fine chemicals, Boisar, Thane. Potassium dihydrogen phosphate and disodium hydrogen phosphate (ExcelR grade) were purchased from Qualigens fine chemicals, Mumbai.

3.2.3 APPARATUS

Cyclomixer and Centrifuge (Remi Equipments, Mumbai), electrically heated thermostatically controlled water bath (Superfit Equipments, Mumbai), Vibronics Ultrasonic cleaner 120W (Vibronics Co. Pvt. Ltd., Mumbai),

Shimadzu UV-1601 UV-Visible spectrophotometer (Shimadzu Corporation, Japan).

3.2.4 ESTIMATION OF PHOSPHATIDYL CHOLINE IN LIPOSOMES

The Stewart assay (Stewart, 1980) was used for estimating phosphatidyl choline in liposomes. This method utilizes the ability of phospholipids to form a complex with ammonium ferrothiocyanate in organic solution.

3.2.4.1 Solutions

1. Ammonium ferrothiocyanate solution (0.1M) was prepared by dissolving 27.03g of ferric chloride hexahydrate and 30.4g of ammonium thiocyanate in double distilled water and making up the volume of the resulting solution to 1 litre.
2. Stock solution of phosphatidyl choline in chloroform (0.1mg/ml) was prepared by dissolving 50mg of phosphatidyl choline in 10ml of chloroform. 2ml of this solution was diluted 100 times to yield a solution of the required concentration.
3. Saturated sodium chloride solution: Sodium chloride was dissolved in distilled water with heating to form a supersaturated solution. This solution was then cooled to room temperature and filtered to give the required saturated solution.

3.2.4.2 Principle

Of the many methods that exist for measuring phospholipids, those based on analysis of the phosphorus content appeared to be most favored. This requires acid digestion of the phospholipids and colorimetric determination of inorganic phosphate formed. This happens to be a sensitive method, but lengthy. Phosphatidyl choline (PC) estimation was done by Stewart assay method (Stewart, 1980). In this method, the ability of PC to form complex with Ammonium ferrothiocyanate in organic solution was utilized.

3.2.4.3 Procedure for calibration curve

Suitable aliquots (0.1 –1.5 ml) of the stock solution of phosphatidyl choline were transferred to 10ml centrifuge tubes. Appropriate quantities of chloroform were then added such that the total volume of the contents of the tubes was 3ml. To each tube, 2ml of ammonium ferrothiocyanate

solution (0.1M) was then added. The contents of each tube were mixed by vigorous vortexing on a cyclomixer for 15sec. The tubes were then spun for 5min at 1800 rpm in a tabletop centrifuge. The lower, organic colored layer was then removed using a syringe and long needle (18 gauge) and transferred to a test tube. The absorbance of these solutions was measured at 485 nm on a Shimadzu 1601 UV-Visible spectrophotometer with glass cells of 10mm path length using a blank prepared in the same manner omitting the phospholipids.

The above procedure was repeated six times. Mean absorbance values along with the regressed values (method of least squares), the calibration curves and the regression equation obtained are shown in table 3.1 and figure 3.1. The optical characteristics of the colored solutions are shown in table 3.2.

Table 3.1 Calibration curve for estimation of phosphatidyl choline (Stewart assay)

Concentration (µg/ml)	Mean Absorbance* ± S.E.M	Regressed value
10	0.116±0.001	0.126
20	0.261±0.005	0.252
30	0.370±0.003	0.375
40	0.506±0.003	0.500
60	0.756±0.003	0.750
80	0.992±0.002	0.999

Regression equation** Y= 0.0125X + 0.001

Correlation coefficient = 0.9993

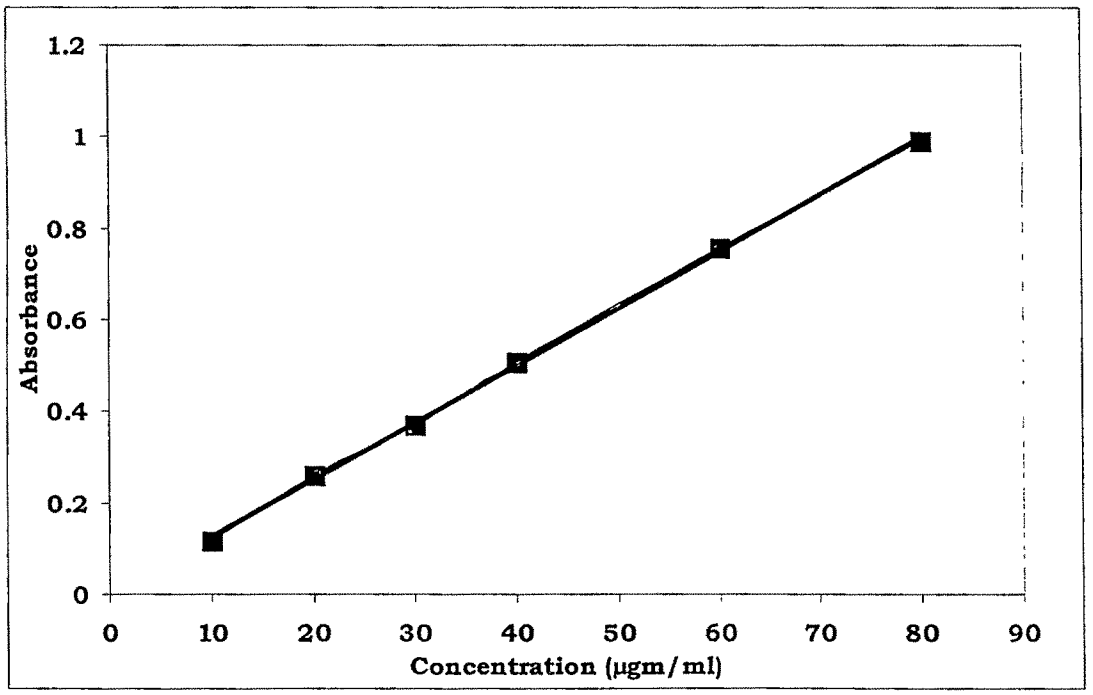
*Mean of 6 values

**n=36.

Table 3.2 Optical characteristics for ammonium ferrothiocyanate-phosphatidyl choline complex in chloroform

Characteristic	Value
Absorption maxima (nm)	485
Beer's law limits (µg/ml)	10-80
Apparent molar absorptivity (l mol^{-1} cm^{-1})	9737
Sandell's sensitivity coefficient (S) (µg/cm^2/0.001 abs unit)	$8.11*10^{-5}$

Figure 3.1 Calibration plot for the estimation of phosphatidyl choline



3.2.4.4 Stability and selectivity

Stability of the colored solutions, prepared above for the calibration curve of phosphatidyl choline, was ascertained by observing the changes in absorbance of the solutions over a period of 4 h. The selectivity of the method for phosphatidyl choline was investigated by carrying out the procedure detailed above in the presence of potential interferences such as cholesterol, cyclosporine, leuprolide acetate, DNA, etc., at the levels at which these materials were included in the liposomes.

3.2.4.5 Estimation of phosphatidyl choline from liposomes/ supernatant

The Bligh-Dyer two-phase extraction method (New, 1990a) was modified for estimating phosphatidyl choline from liposomes. Briefly, 0.1ml of liposomal dispersion or 0.5ml of the supernatant was taken in a centrifuge tube and to this 1ml of saturated sodium chloride solution was added. 2ml of chloroform was then added to the contents followed by vigorous vortexing on a cyclomixer for 30 sec and centrifugation at 1800 rpm for 5 min in a tabletop centrifuge. The lower chloroform layer was separated using a syringe and needle (18 gauge) and passed over a bed of anhydrous sodium sulphate into a 10ml volumetric flask. The process was repeated with a further 2ml and 1ml of chloroform. The chloroform layers were then pooled and the volume made up to 10ml with chloroform. To 0.5ml of this chloroform extract in a centrifuge tube, 2.5ml of chloroform and 2ml of 0.1M ammonium ferrothiocyanate solution was added. The contents were then subjected to the same procedure as detailed above for the standards (Section 3.2.4.3). Duplicate estimations were performed and the mean absorbance was used to determine the amount of phosphatidyl choline in the liposomes or supernatant using the regression equation.

3.2.5 ESTIMATION OF CHOLESTEROL IN LIPOSOMES

The Zlatkis, Zak and Boyle's method was used for estimating cholesterol in liposomes. This method utilizes the ability of cholesterol in acetic acid to form a complex with ferric chloride and sulphuric acid (Zlatkis et. al., 1953).

3.2.5.1 Solutions

1. Ferric chloride solution: A 0.05% w/v solution of ferric chloride hexahydrate in glacial acetic acid was prepared by dissolving 50mg of ferric chloride hexahydrate in 100ml glacial acetic acid.
2. Stock solution of cholesterol: A 0.5mg/ml solution of cholesterol in glacial acetic acid was prepared by dissolving 25mg of cholesterol in 50ml of glacial acetic acid.
3. Saturated sodium chloride solution: It was prepared in the same manner as for the estimation of phosphatidyl choline from liposomes (Section 3.2.4.1)

3.2.5.2 Principle

The Zlatkis, Zak and Boyle method (Zlatkis et. al., 1953) was used for estimating cholesterol in liposomes. Here, cholesterol in acetic acid forms a colored complex with ferric chloride and sulphuric acid, which exhibits maximum absorbance at 560nm.

3.2.5.3 Procedure for calibration curve

Suitable aliquots of the stock solution of cholesterol (0.1 to 1 ml) were transferred accurately into 10ml volumetric flasks. To each flask, 4ml of ferric chloride solution and 4ml of concentrated sulphuric acid was added. The contents were mixed, made up to the volume with glacial acetic acid and allowed to stand for 30 min. The absorbance of the resulting colored solutions of the complex, formed between cholesterol, ferric chloride and sulphuric acid was measured at 550nm using Shimadzu 1601 UV-Visible spectrophotometer with glass cells of 10mm path length, against a blank prepared in the same manner as the standard solutions except cholesterol.

The above procedure was repeated six times. The experimental data along with the results of the statistical evaluation of the data and calibration curves obtained are shown in table 3.3 and figure 3.2. The optical characteristics for the above solutions of the complex formed are tabled in table 3.4.

3.2.5.4 Stability and selectivity

Stability of the solutions of the complex, prepared for obtaining the calibration curve of cholesterol, was ascertained by observing the changes in their absorbances over a period of 24 h.

Cholesterol was estimated in the presence of phosphatidyl choline, cyclosporine, leuprolide acetate, DNA, etc., at the same concentrations at which these materials were included in the liposomes to ascertain the selectivity of the method.

3.2.5.5 Estimation of cholesterol from liposomes/ supernatant

1ml of the chloroform extract, obtained as described before in the estimation of phosphatidyl choline form liposomes or supernatant (Section 3.2.4.5) was taken in a 10ml volumetric flask and evaporated to dryness by heating at 90°C in a thermostatically controlled, electrically heated water bath. The dried contents were then subjected to the same procedure as discussed previously (Section 3.2.5.3). The amount of cholesterol in the liposomes or supernatant was then obtained using the regression equation of the calibration curve (table 3.3).

Table 3.3 Calibration curve for estimation of cholesterol (Zlatkis, Zak and Boyle's method)

Concentration (µg/ml)	Mean Absorbance* ± S.E.M	Regressed value
10	0.175±0.002	0.183
20	0.365±0.004	0.3593
30	0.542±0.001	0.5363
40	0.717±0.002	0.713
50	0.879±0.004	0.889
60	1.068±0.006	1.066

Regression equation** Y= 0.0177X+ 0.0061

Correlation coefficient = 0.9995

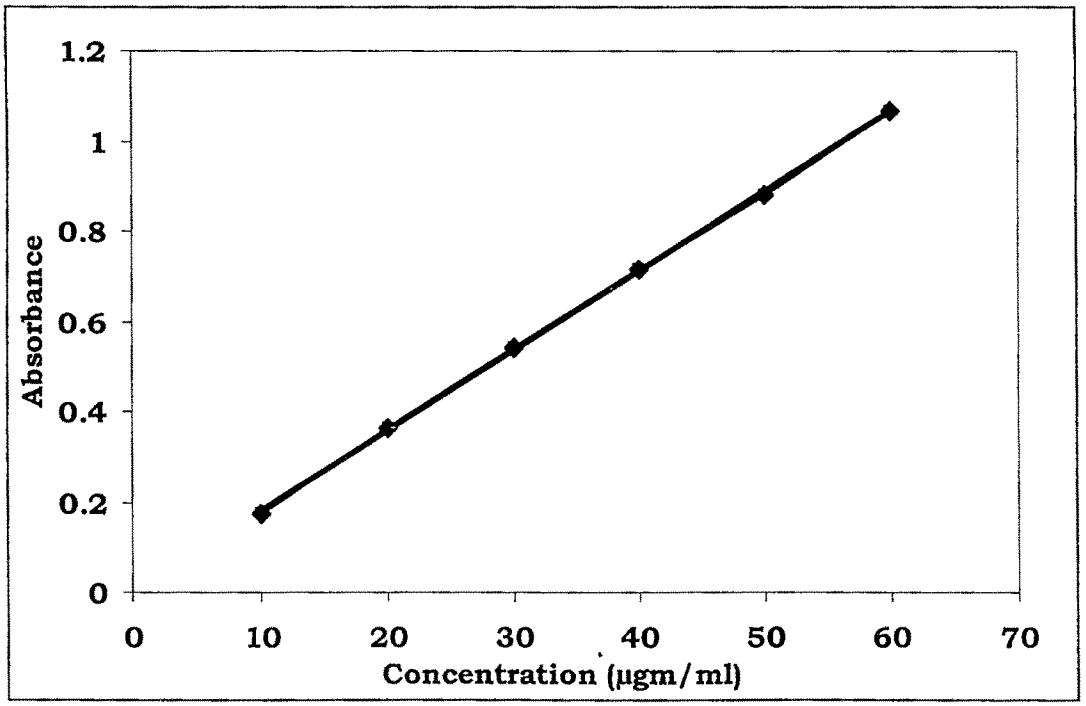
*Mean of 6 values

**n=36.

Table 3.4 Optical characteristics for ammonium ferrothiocyanate-phosphatidyl choline complex in chloroform

Characteristic	Value
Absorption maxima (nm)	550
Beer's law limits (µg/ml)	10-60
Apparent molar absorptivity (l mol^{-1} cm^{-1})	6995
Sandell's sensitivity coefficient (S) (µg/cm^2/0.001 abs unit)	$5.53*10^{-5}$

Figure 3.2 Calibration plot for the estimation of Cholesterol



3.2.6 ESTIMATION OF CYCLOSPORINE IN LIPOSOMES

Cyclosporine in Tetrahydrafuran/methanol (1:1) shows absorbance in the ultraviolet region of the electromagnetic spectrum (Adler et.al., 1992).

3.2.6.1 Solutions

Stock solution of cyclosporine (100µg/ml) was prepared by dissolving 10mg of cyclosporine in 100ml of Tetrahydrafuran/methanol (1:1).

3.2.6.2 Procedure for calibration curve

Suitable aliquots (0.5ml –3.0ml) of the stock solution of were pipetted into 10ml volumetric flasks and the volume was made up to 10ml with Tetrahydrafuran/methanol (1:1) to give final concentrations of 10, 20, 30, 40, 50 and 60µg/ml. The solutions were shaken well and their absorbances measured at 238nm using Tetrahydrafuran/methanol (1:1) as blank on a Shimadzu 1601 UV-Visible spectrophotometer. The above procedure was repeated six times. Table 3.5 tabulated the raw and regressed data (method of least squares) so obtained whereas Table 3.6 contains the optical characteristics for the solution of cyclosporine in Tetrahydrafuran / methanol (1:1). Absorptivity scan over the UV wavelength range between 200 and 400 nm for 100µg/ml solution of cyclosporine in Tetrahydrafuran/methanol (1:1) and the calibration curves obtained are shown in Figure 3.3 and 3.4.

3.2.6.3 Stability and selectivity

Stability of the solutions of cyclosporine in Tetrahydrafuran/methanol (1:1), used for preparing the calibration curve, was ascertained by observing the changes in their absorbance at the analytical wavelength, over a period of 48h at room temperature.

3.2.6.4 Estimation of cyclosporine from liposomes/ supernatant

To 0.1ml of liposomes or 0.5ml of supernatant in a 10ml volumetric flask, Tetrahydrafuran/methanol (1:1) was added to break the liposomes and the volume was made up with Tetrahydrafuran/methanol (1:1). The absorbance was measured at 238nm against a blank comprising of empty liposomes diluted with Tetrahydrafuran/methanol (1:1) in the similar manner. Triplicate estimations were made and the mean absorbances were determined. The amount of cyclosporine in the liposomes or supernatant was then obtained using the regression equation.

Table 3.5 Calibration curve for cyclosporine in Tetrahydrafuran /methanol (1:1)

Concentration (µg/ml)	Mean Absorbance* ± S.E.M	Regressed Value
10	0.076±0.003	0.074
20	0.137±0.003	0.138
30	0.201±0.005	0.203
40	0.26±0.005	0.267
50	0.335±0.003	0.335
60	0.396±0.002	0.395

Regression equation++: Y = 0.0064X + 0.0103

Correlation co efficient = 0.999

*Mean of 6 values

++n=30

Table 3.6 Optical characteristics for cyclosporine in Tetrahydrafuran /methanol (1:1)

Characteristic	Value
Absorption maxima (nm)	238*
Beer's law limits (µg/ml)	10-60
Apparent molar absorptivity (l/mol/cm)	8053
Sandell's sensitivity coefficient (S) (µg/cm²/0.001 abs unit)	1.5×10^{-4}

* Analytical wavelength

Figure 3.3 Absorptivity scan of cyclosporine in THF/Methanol

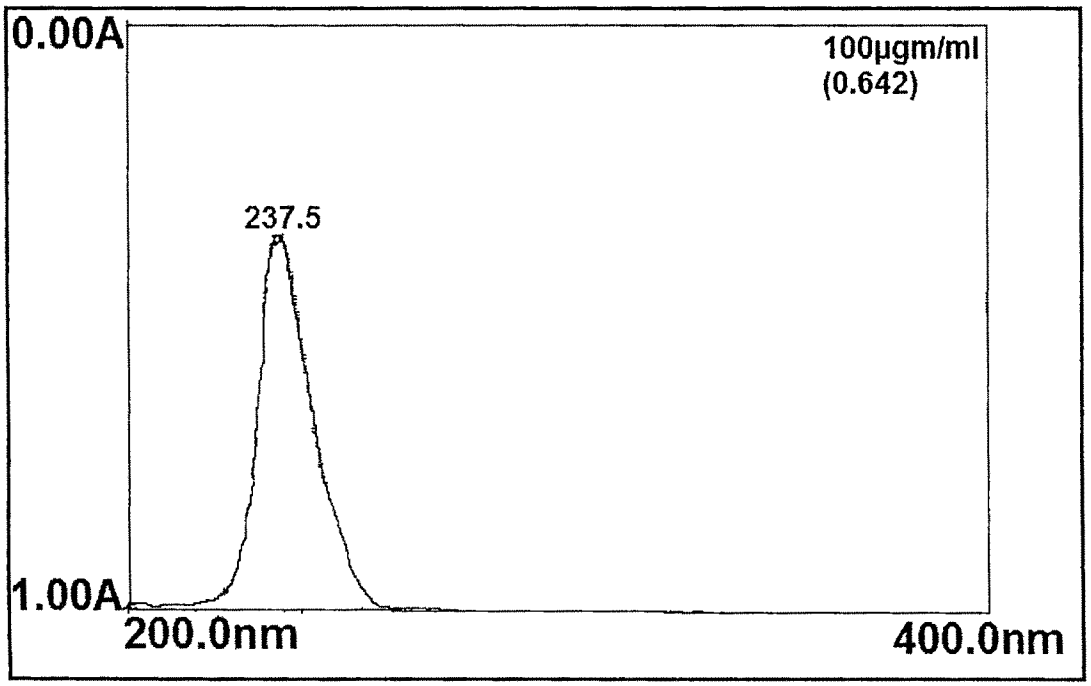
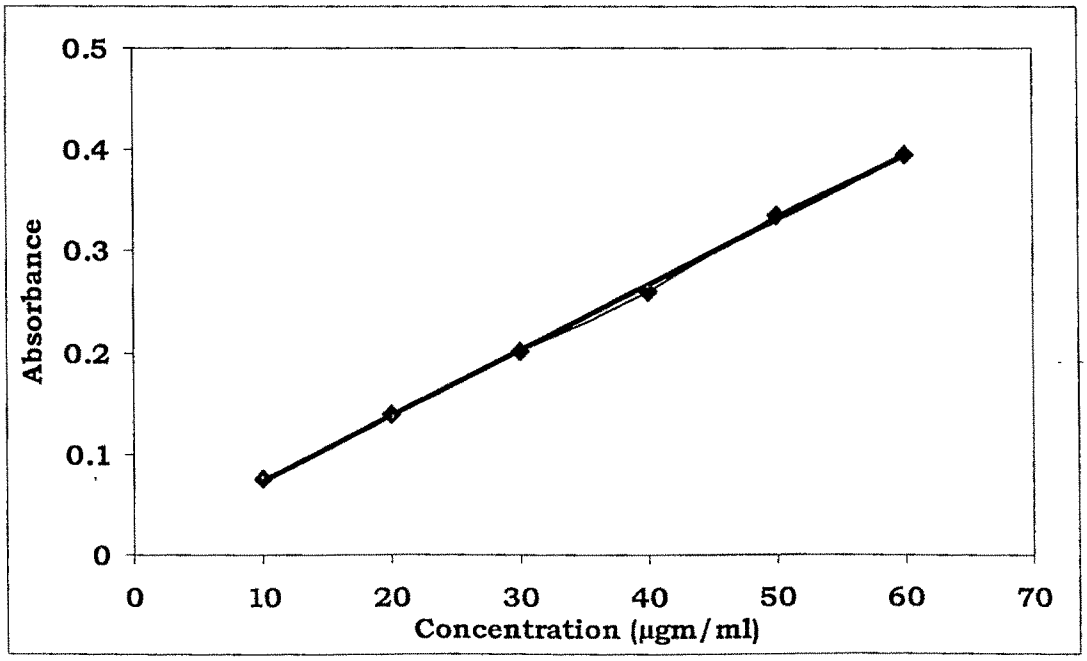


Figure 3.4 Calibration plot for the estimation of cyclosporine



3.2.7 ESTIMATION OF LEUPROLIDE ACETATE IN 0.1N SODIUM HYDROXIDE

Leuprolide acetate in 0.1N sodium hydroxide (0.1N NaOH) shows strong absorbance in the ultraviolet region of the electromagnetic spectrum (Adjei and Hsu, 1993).

3.2.7.1 Solutions

Stock solution of leuprolide acetate (100µg/ml) was prepared by dissolving 5mg of leuprolide acetate in 50ml of 0.1N sodium hydroxide).

3.2.7.2 Procedure for calibration curve

Suitable aliquots (0.5 –3ml) of the stock solution of leuprolide acetate were pipetted into 5ml volumetric flasks and the volume was made up to 5ml with 0.1N NaOH to give final concentrations of 10, 20, 30, 40, 50and 60µg/ml. The solutions were shaken well and their absorbances measured at 282nm using 0.1N NaOH as blank. The above procedure was repeated six times. Table 3.7 tabulated the raw and regressed data (method of least squares) so obtained whereas table 3.8 contains the optical characteristics for the solution of leuprolide acetate in 0.1N NaOH. Absorptivity scans over the UV wavelength range between 220 and 400nm for 50µg/ml solution of leuprolide acetate in 0.1N NaOH and the calibration curves are shown in figure 3.5 and 3.6.

3.2.7.3 Stability and selectivity

Stability of the solutions of leuprolide acetate in 0.1N NaOH, used for preparing the calibration curve, was ascertained by observing the changes in their absorbance at the analytical wavelength, over a period of 48h at room temperature.

The above method for estimating leuprolide acetate was carried out in the presence of phosphatidyl choline, cholesterol, α-tocopherol and other components of the liposomes to ascertain the selectivity of the method.

3.2.7.4 Estimation of leuprolide acetate from liposomes/ supernatant

The unentrapped drug was removed from the liposomal suspension by ficoll- gradient centrifugation (New, 1990). 0.5ml of liposomal suspension was mixed with 1ml of 30% ficoll solution (in saline). It was transferred to

an ultracentrifuge tube; 3ml of 10% ficoll solution was layered gently on top of the above mixture. The upper ficoll layer was covered with a layer of buffered saline, centrifuged (Remi, C-24, India) at 15,000 rpm for 30 min. The unentrapped drug present in the lowest ficoll layer was diluted appropriately using 0.1N sodium hydroxide and the absorbance was measured at 282nm on Shimadzu 1601 UV- Visible Spectrophotometer. The liposomes were collected at the interface between the saline and 10% ficoll layers.

The drug present in the liposomes was estimated by Modified Bligh-Dyer extraction method. 0.5ml of liposome suspension, 2ml of chloroform, 1ml of methanol and 1ml of saturated sodium chloride solution were taken in a centrifuge tube, the mixture was spun at 1000 rpm for about 10 minutes. The aqueous layer was removed and the drug content was estimated by UV-Visible Spectrophotometer at 282nm after dilution with 0.1N sodium hydroxide.

Table 3.7 Calibration curve for leuprolide acetate in 0.1N NaOH

Concentration (µg/ ml)	Mean Absorbance* ± S.E.M	Regressed Value
10	0.059±0.002	0.059
20	0.112±0.002	0.111
30	0.162±0.001	0.163
40	0.217±0.001	0.215
50	0.265±0.003	0.267
60	0.324±0.002	0.319

Regression equation^{++}: Y = 0.0053x + 0.0059

Correlation co efficient = 0.9995

*Mean of 6 values

$^{++}$n=36

Table 3.8 Optical characteristics for leuprolide acetate in 0.1N NaOH

Characteristic	Value
Absorption maxima (nm)	242, 282*
Beer's law limits (µg/ml)	10-60
Apparent molar absorptivity (l/mol/cm)	6649
Sandell's sensitivity coefficient (S) (µg/cm²/0.001 abs unit)	$1.9*10^{-4}$

*Analytical wavelength

Figure 3.5 Absorptivity scan of leuprolide acetate in 0.1N NaOH (50µg/ml)

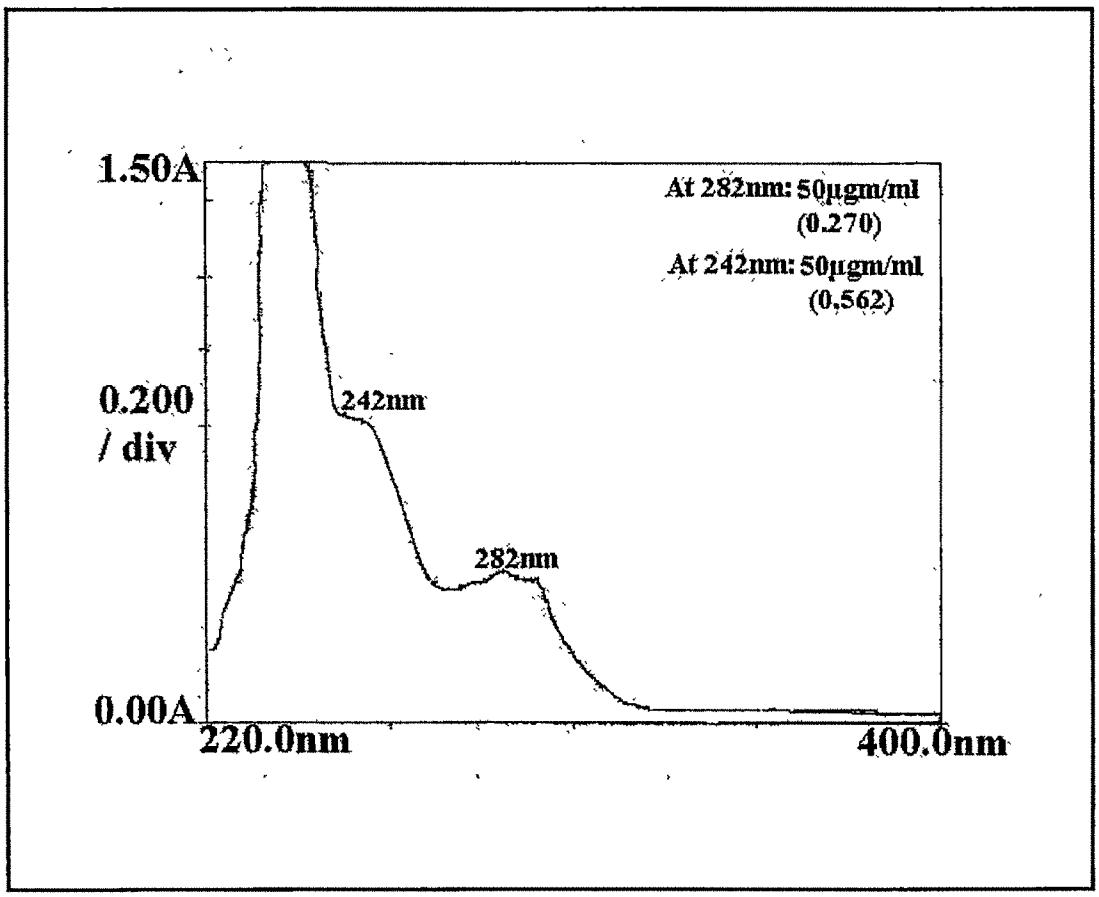
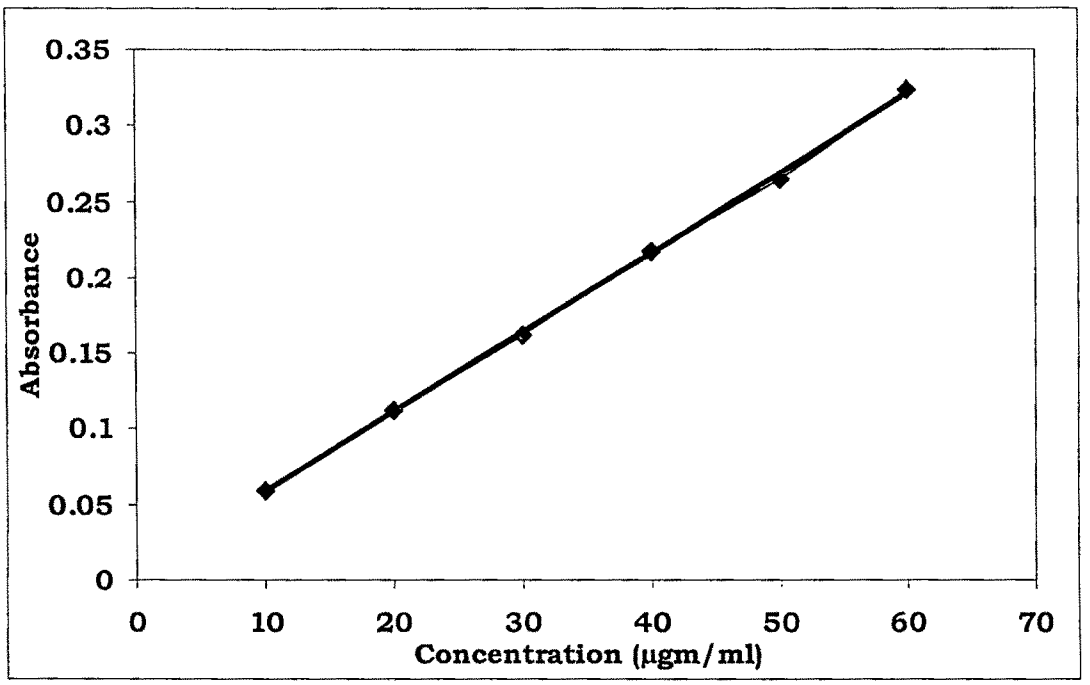


Figure 3.6 Calibration curve for leuprolide acetate in 0.1N NaOH



3.2.8 ESTIMATION OF DNA IN TRIS BUFFER pH 8.0

DNA in Tris buffer pH 8.0 shows strong absorbance in the ultraviolet region of the electromagnetic spectrum (Middaugh et al., 1997).

3.2.8.1 Solutions

Stock solution of DNA (100µg/ml) was prepared by dissolving 5mg of DNA in 50ml of Tris buffer (pH 8.0, 10mM of Tris and 1mM of EDTA).

3.2.8.2 Procedure for calibration curve

Suitable aliquots (0.02 –1.2ml) of the stock solution of DNA were pipetted into 5ml volumetric flasks and the volume was made up to 5ml with Tris buffer to give final concentrations of 4, 8, 12, 16, 20 and 22µg/ml. The solutions were shaken well and their absorbances measured at 260nm using Tris buffer as blank. The above procedure was repeated six times. Table 3.9 tabulated the raw and regressed data (method of least squares) so obtained whereas table 3.10 contains the optical characteristics for the solution of DNA in Tris buffer. Absorptivity scans over the UV wavelength range between 200 and 400nm for 20µg/ml solution of DNA in Tris buffer and the calibration curves are shown in figure 3.7 and 3.8.

3.2.8.3 Stability and selectivity

Stability of the solutions of DNA in Tris buffer, used for preparing the calibration curve, was ascertained by observing the changes in their absorbance at the analytical wavelength, over a period of 48h at room temperature.

The above method for estimating DNA was carried out in the presence of phosphatidyl choline, cholesterol, α-tocopherol and other components of the liposomes to ascertain the selectivity of the method.

3.2.8.4 Estimation of DNA from liposomes/supernatant

The unentrapped DNA was removed from the liposomal suspension by ficoll- gradient centrifugation (New, 1990). 0.5ml of liposomal suspension was mixed with 1ml of 30% ficoll solution (in saline). It was transferred to an ultracentrifuge tube; 3ml of 10% ficoll solution was layered gently on top of the above mixture. The upper ficoll layer was covered with a layer of buffered saline, centrifuged (Remi, C-24, India) at 15,000 rpm for 30 min. The unentrapped DNA present in the lowest ficoll layer was diluted appropriately using tris buffer and the absorbance was measured at 260nm on Shimadzu 1601 UV- Visible Spectrophotometer. The liposomes were collected at the interface between the saline and 10% ficoll layers.

The DNA present in the liposomes was estimated by Modified Bligh-Dyer extraction method. 0.5ml of liposome suspension, 2ml of chloroform, 1ml of methanol and 1ml of saturated sodium chloride solution were taken in a centrifuge tube, the mixture was spun at 1000 rpm for about 10 minutes. The aqueous layer was removed and the DNA content was estimated by UV-Visible Spectrophotometer at 260nm after dilution with tris buffer.

Table 3.9 Calibration curve for DNA in Tris buffer

Concentration (µg/ ml)	Mean Absorbance* ± S.E.M	Regressed Value
4	0.077±0.002	0.078
8	0.160±0.002	0.165
12	0.249±0.001	0.252
16	0.332±0.002	0.339
20	0.421±0.004	0.427
22	0.465±0.002	0.470

Regression equation^{++}: Y=0.0209x-0.0052

Correlation co efficient = 0.9995

*Mean of 6 values

$^{++}$n=36

Table 3.10 Optical characteristics for DNA in Tris buffer.

Characteristics	Value
Absorption maxima (nm)	260*
Beer's law limits (µg/ml)	4-22

*Analytical wavelength

Figure 3.7 Absorptivity of DNA in Tris buffer (20µgm/ml)

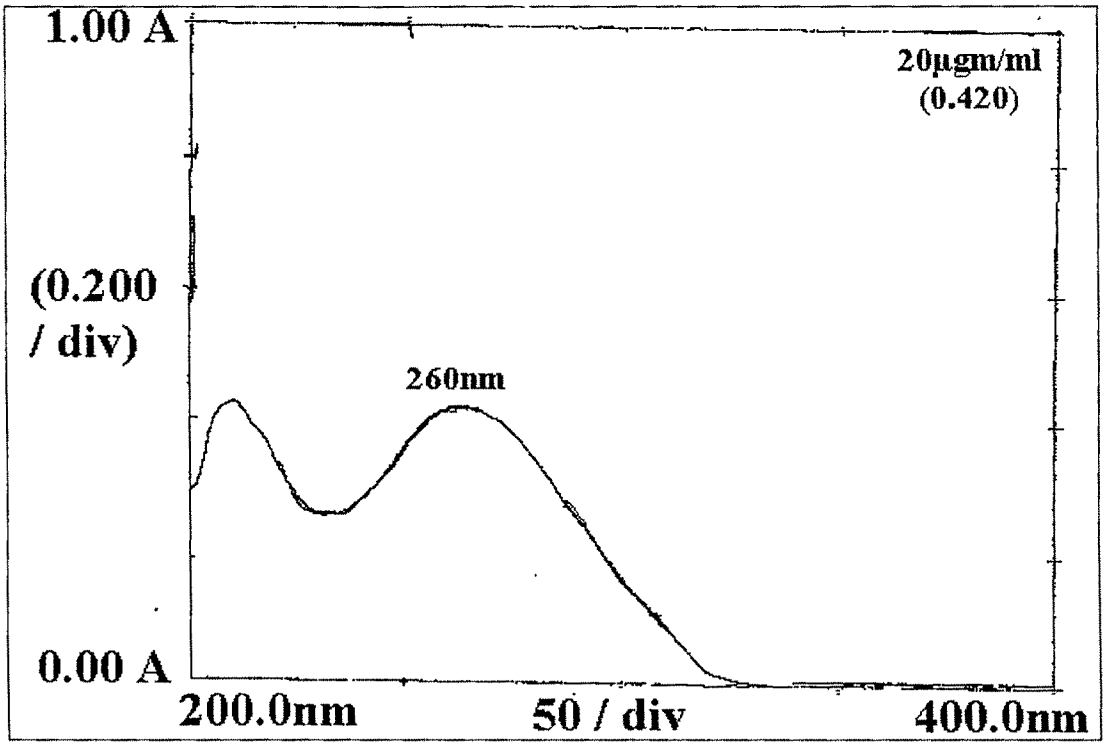
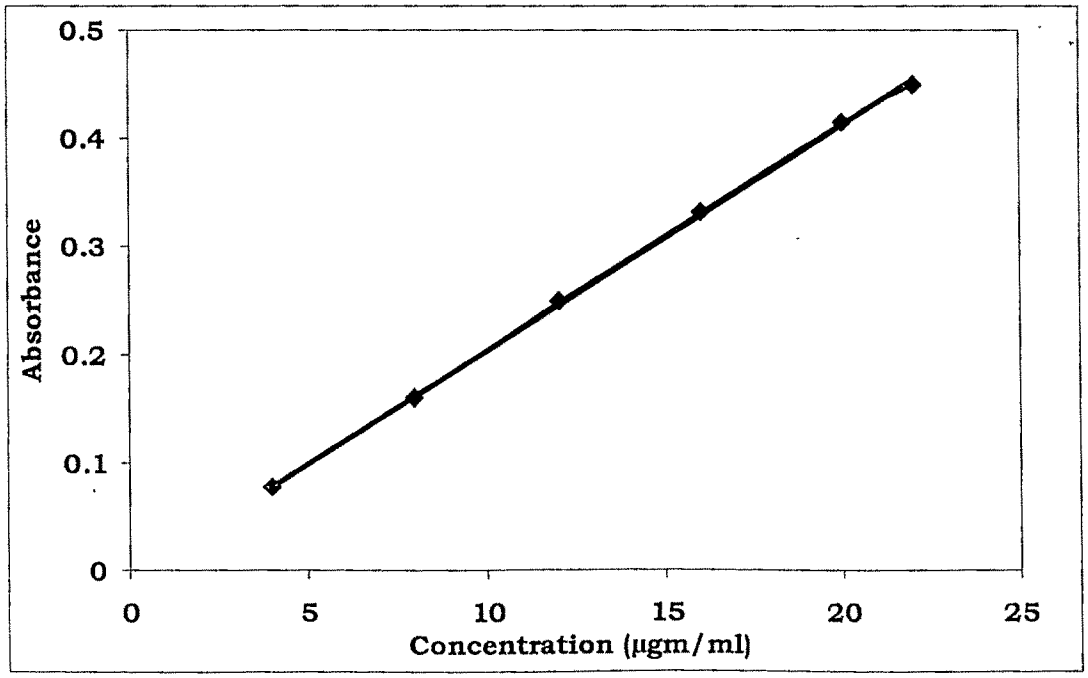


Figure 3.8 Calibration curve for DNA in Tris buffer



3.2.9 ESTIMATION OF LEUPROLIDE ACETATE IN PHOSPHATE BUFFER SALINE (PBS)

Leuprolide acetate in PBS shows strong absorbance in the ultraviolet region of the electromagnetic spectrum.

3.2.9.1 Solutions

Stock solution of leuprolide acetate (100µg/ml) was prepared by dissolving 5mg of leuprolide acetate in 50ml of PBS, pH 7.4 (The Indian Pharmacopoeia, 1985).

3.2.9.2 Procedure for calibration curve

Suitable aliquots (0.5 –3ml) of the stock solution of leuprolide acetate were pipetted into 5ml volumetric flasks and the volume was made up to 5ml with PBS to give final concentrations of 10, 20, 30, 40, 50 and 60µg/ml. The solutions were shaken well and their absorbances measured at 280nm using PBS as blank. The above procedure was repeated six times. Table 3.11 tabulated the raw and regressed data (method of least squares) so obtained whereas table 3.12 contains the optical characteristics for the solution of leuprolide acetate in PBS. Absorptivity scans over the UV wavelength range between 220 and 400nm for 100µg/ml solution of leuprolide acetate in PBS and the calibration curves obtained are shown in figure 3.9 and 3.10.

3.2.9.3 Stability and selectivity

Stability of the solutions of leuprolide acetate in PBS, used for preparing the calibration curve, was ascertained by observing the changes in their absorbance at the analytical wavelength, over a period of 48h at room temperature.

The above method for estimating leuprolide acetate was carried out in the presence of phosphatidyl choline, cholesterol, α-tocopherol and other components of the liposomes to ascertain the selectivity of the method.

Table 3.11 Calibration curve for leuprolide acetate in PBS

Concentration (µg/ml)	Mean Absorbance* ± S.E.M	Regressed Value
10	0.050±0.001	0.046
20	0.077±0.001	0.079
30	0.112±0.001	0.113
40	0.144±0.001	0.147
50	0.179±0.002	0.18
60	0.219±0.003	0.213

Regression equation^{++}: Y = 0.0034x + 0.0119

Correlation co efficient = 0.9971

*Mean of 6 values

$^{++}$n=36

Table 3.12 Optical characteristics for leuprolide acetate in PBS

Characteristics	Value
Absorption maxima (nm)	280
Beer's law limits (µg/ml)	10-60
Apparent molar absorptivity (l/mol/cm)	4593
Sandell's sensitivity coefficient (S) (µg/cm^2/0.001 abs unit)	1.38*10^{-4}

*Analytical wavelength

Figure 3.9 Absorptivity scan of leuprolide acetate in PBS (100µg/ml)

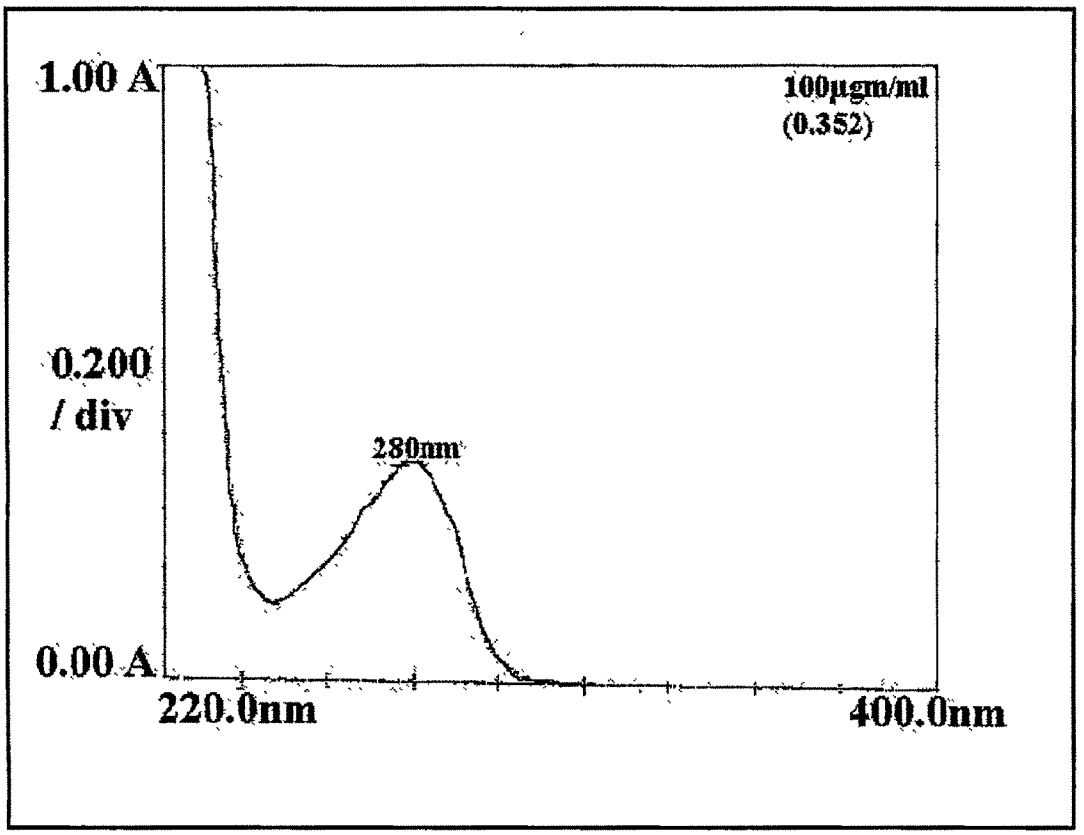
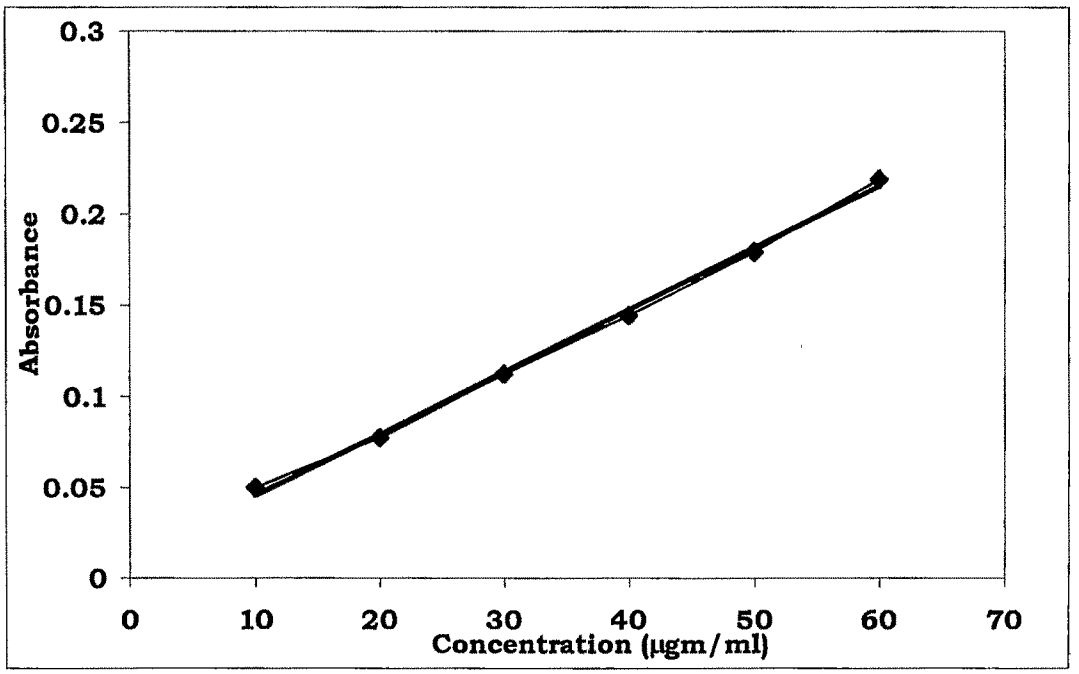


Figure 3.10 Calibration curve for leuprolide acetate in PBS.



3.2.10 ESTIMATION OF DNA IN PBS

DNA in PBS shows strong absorbance in the ultraviolet region of the electromagnetic spectrum.

3.2.10.1 Solutions

Stock solution of DNA (100µg/ml) was prepared by dissolving 5mg of DNA in 50ml of PBS, pH 7.4.

3.2.10.2 Procedure for calibration curve

Suitable aliquots (0.02 –1.2ml) of the stock solution of DNA were pipetted into 5ml volumetric flasks and the volume was made up to 5ml with PBS to give final concentrations of 4, 8, 12, 16, 20 and 22µg/ml. The solutions were shaken well and their absorbances measured at 260nm using PBS as blank. The above procedure was repeated six times. Table 3.13 tabulated the raw and regressed data (method of least squares) so obtained whereas table 3.14 contains the optical characteristics for the solution of DNA in PBS. Absorptivity scans over the UV wavelength range between 200 and 400nm for 20µg/ml solution of DNA in PBS and the calibration curves obtained are shown in figure 3.11 and 3.12.

3.2.10.3 Stability and selectivity

Stability of the solutions of DNA in PBS, used for preparing the calibration curve, was ascertained by observing the changes in their absorbance at the analytical wavelength, over a period of 48h at room temperature.

The above method for estimating DNA was carried out in the presence of phosphatidyl choline, cholesterol, α-tocopherol and other components of the liposomes to ascertain the selectivity of the method.

Table 3.13 Calibration curve for DNA in PBS

Concentration (µg/ ml)	Mean Absorbance* ± S.E.M	Regressed Value
4	0.085±0.002	0.086
8	0.178±0.003	0.178
12	0.27±0.002	0.270
16	0.362±0.001	0.362
20	0.460±0.002	0.454
22	0.495±0.004	0.500

Regression equation++: Y=0.023x-0.0063

Correlation co efficient = 0.9995

*Mean of 6 values ++n=36

Table 3.14 Optical characteristics for DNA in PBS

Characteristics	Value
Absorption maxima (nm)	260*
Beer's law limits (µg/ml)	4-22

*Analytical wavelength

Figure 3.12 Calibration curve for DNA in PBS

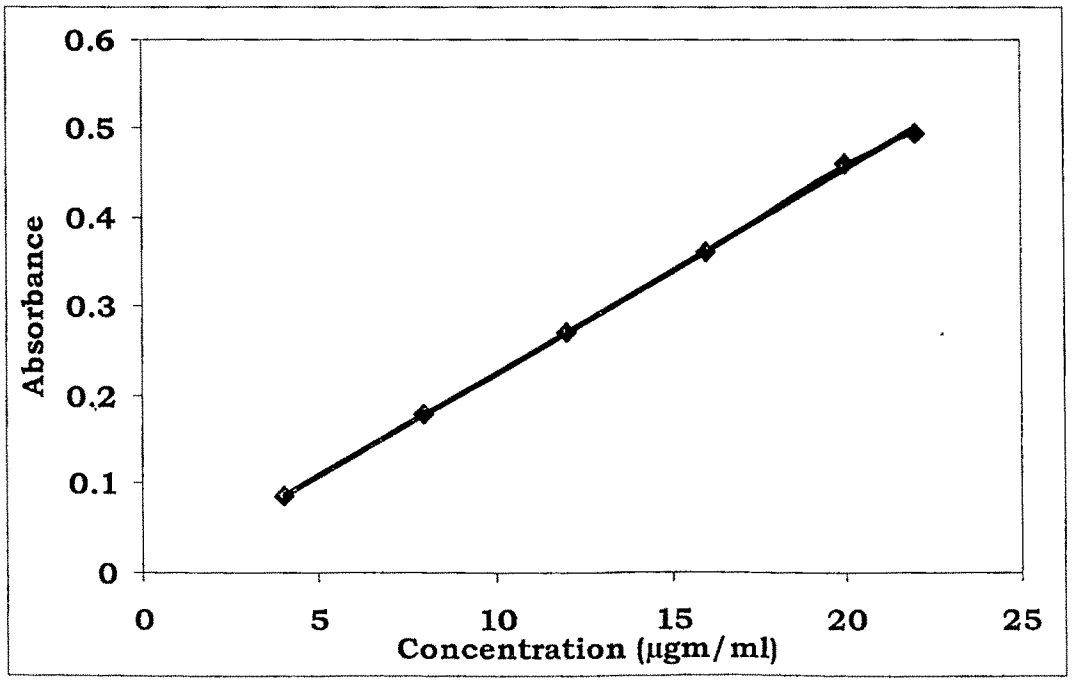
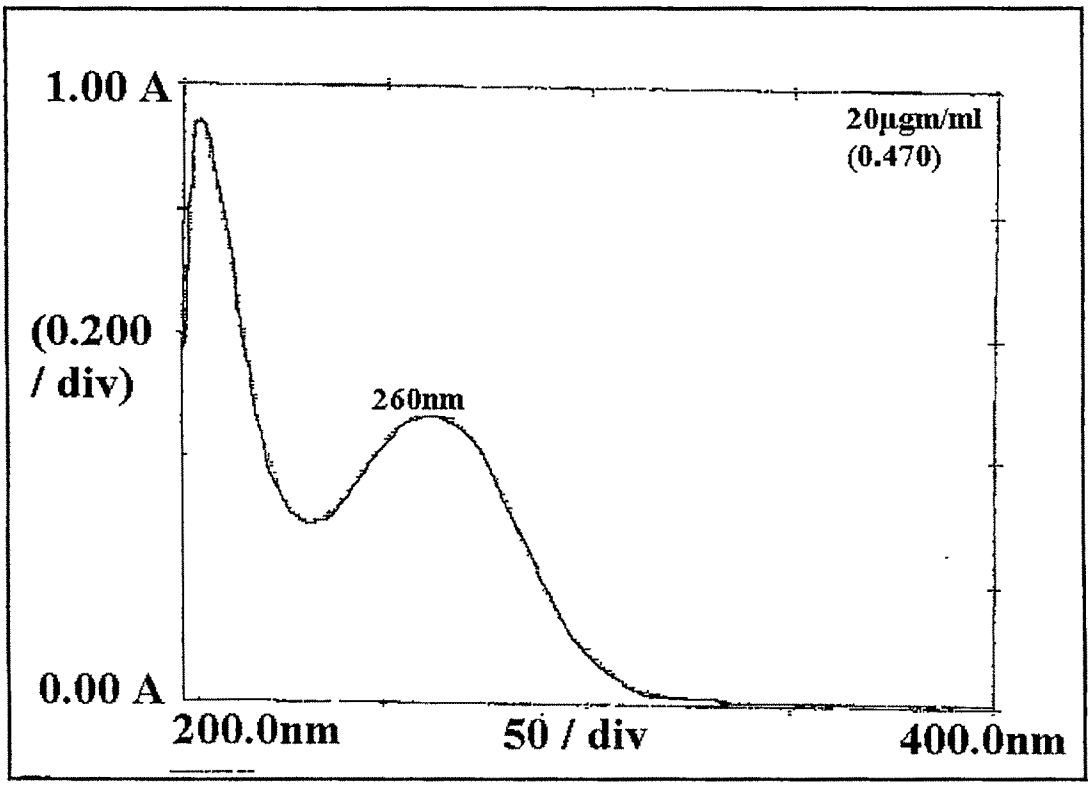


Figure 3.11 Absorptivity scan of DNA in PBS (20µg/ml)



3.2.11 ESTIMATION OF POLYETHYLENE GLYCOL DERIVATIVES IN LIPOSOMES

The estimation of polyethylene glycol derivatives [methoxy polyethylene glycol 5000 activated with cyanuric chloride-phosphatidylethanolamine conjugate (mPEG5000-CC-PE), methoxy polyethylene glycol 2000 activated with cyanuric chloride-phosphatidyl ethanolamine conjugate (mPEG2000-CC-PE)] were done using the method reported (Shimada et.al., 2000).

This method is based on the spectrophotometric determination of complexes of polyethylene glycols with sodium ions after their extraction as picrates, into 1,'2-dichloromethane, and provided a sensitive tool for determination of PEG-lipids.

3.2.11.1 Solutions

To a solution containing 0.1 mol sodium hydroxide, 280 g of sodium nitrate (3.3 mol) and 4.58 g of picric acid (0.02mol) were added and the final

volume of the solution was adjusted to 1000 ml. After sonication, the solution was filtered off and stored in a refrigerator at 5°C.

Stock solution of polyethylene glycol derivatives (mPEG5000-CC-PE/ mPEG2000-CC-PE) (1mg/ml) was prepared by dissolving 100mg of polyethylene glycol (mPEG5000-CC-PE/mPEG2000-CC-PE) in 100ml of distilled water.

3.2.11.2 Procedure for calibration curve

Suitable aliquots (0.5 –5ml) of the stock solution of polyethylene glycol derivatives were pipetted into 5ml volumetric flasks and the volume was made up to 5ml with distilled water to give final concentrations of 10, 20, 40, 60, 80 and 100µg/ml. To the 5ml of above sample solution, 10ml of sodium nitrate picrate mixture and 5ml of dichloromethane was added. The solution was shaken well and the absorbance was measured at 378nm on a Shimadzu 1601 UV-Visible spectrophotometer. The above procedure was repeated six times. Tables 3.15 and 3.17 tabulated the raw and regressed data (method of least squares) so obtained whereas tables 3.16 and 3.18 contains the optical characteristics for the solutions of polyethylene glycol derivatives (mPEG5000-CC-PE/mPEG2000-CC-PE) in dichloromethane. The calibration curves obtained for the solutions of polyethylene glycol derivatives (mPEG5000-CC-PE/mPEG2000-CC-PE) in dichloromethane are shown in figure 3.13 and 3.14.

3.2.11.3 Stability and selectivity

Stability of the solutions of polyethylene glycol derivatives, used for preparing the calibration curve, was ascertained by observing the changes in their absorbance at the analytical wavelength, over a period of 24h at room temperature.

The above method for estimating polyethylene glycol derivatives was carried out in the presence of leuprolide acetate, DNA, phosphatidyl choline, cholesterol, α-tocopherol and other components of the liposomes to ascertain the selectivity of the method.

3.2.11.4 Estimation of polyethylene glycol derivatives from liposomes/ supernatant

0.1ml of liposomal suspension or 0.5ml of supernatant was diluted to 5ml with distilled water and was mixed with 10ml of sodium nitrate-picrate solution. To the mixture, 5 ml of 1, 2-dichloromethane was added. After vigorous shaking, the solution was centrifuged at 1500rpm for 10 min and the organic layer was collected and spectrophotometrically measured at 378 nm.

Table 3.15 Calibration curve for mPEG5000-CC-PE

Concentration (µg/ml)	Mean Absorbance* ± S.E.M	Regressed Value
10	0.150±0.014	0.167
20	0.315±0.013	0.301
30	0.442±0.012	0.435
40	0.572±0.016	0.569
50	0.705±0.001	0.703
60	0.839±0.003	0.836

Regression equation++: Y = 0.0136X + 0.0293

Correlation coefficient = 0.9985

*Mean of 6 values

++n=36

Table 3.16 Optical characteristics for mPEG5000-CC-PE

Characteristic	Value
Absorption maxima (nm)	378*
Beer's law limits (µg/ml)	10-60
Apparent molar absorptivity (l/mol/cm)	81986
Sandell's sensitivity coefficient (S) (µg/cm²/0.001 abs unit)	7.09 x 10^{-5}

* Analytical wavelength

Figure 3.13 Calibration curve for mPEG5000-CC-PE

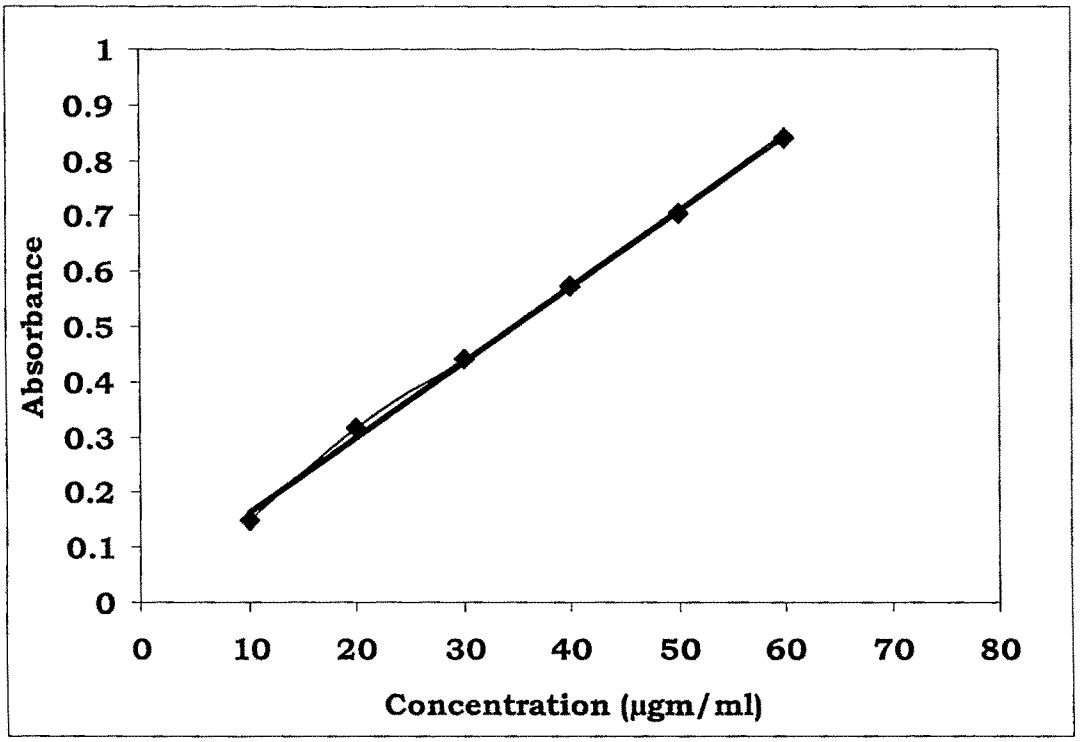


Table 3.17 Calibration curve for mPEG2000-CC-PE

Concentration (µg/ml)	Mean Absorbance* ± S.E.M	Regressed Value
10	0.135±0.002	0.144
20	0.289±0.001	0.278
30	0.410±0.003	0.412
40	0.551±0.002	0.546
50	0.682±0.002	0.680
60	0.809±0.013	0.814

Regression equation++: Y = 0.0134X + 0.0103

Correlation coefficient = 0.9992

*Mean of 6 values

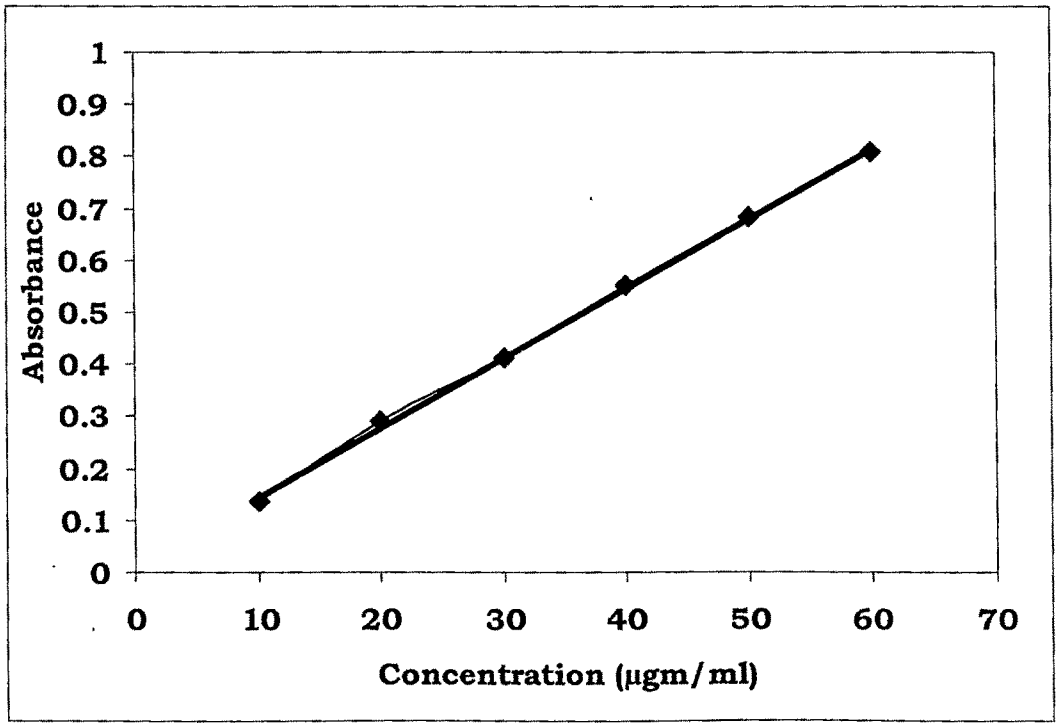
++n=36

Table 3.18 Optical characteristics for mPEG2000-CC-PE

Characteristic	Value
Absorption maxima (nm)	378*
Beer's law limits (µg/ml)	10-60
Apparent molar absorptivity (l/mol/cm)	38315
Sandell's sensitivity coefficient (S) (µg/cm^2/0.001 abs unit)	7.3 x 10^{-5}

*Analytical wavelength

Figure 3.14 Calibration curve for mPEG2000-CC-PE



3.3 RESULTS AND DISCUSSION

3.3.1 ESTIMATION OF PHOSPHATIDYL CHOLINE IN LIPOSOMES

The Stewart assay was used for estimating phosphatidyl choline in liposomes (Stewart, 1980). This method is based on complex formation between ammonium ferrothiocyanate and phospholipids in organic solution. The complex in chloroform exhibits maximum absorbance at 485nm (New, 1990a).

Absorptivity of the complex was calculated as 9737l/mol/cm (table 3.2). A correlation coefficient of 0.9993 (table 3.1) indicated a linear relationship between absorbance and concentration of phosphatidyl choline taken for complex formation. Beer's law was found to be obeyed between 10 –80 µg/ml (table 3.2). This high range is a reflection of the low absorptivity of the complex at the analytical wavelength. The data of regression analysis on the collected data along with the raw data are presented in table 3.1. The regression equation obtained was Y = 0.0125X + 0.001.

The variance of the response variable, S^2_{yx} was obtained as 9.07×10^{-5}. This low value denoted the closeness of experimental points to the least squares line and thus, the low variability in the procedure. This low variability can also be seen from the low value of the standard error of the mean absorbances of the solutions used for obtaining the calibration curve (table 3.1). The variance of the slope, S^2_b was calculated as 2.7×10^{-8}. The value of the slope (0.0125, table 3.1) indicated low sensitivity of the method, a fact supported by the high value of the Sandell's sensitivity coefficient (8.11×10^{-5} µg/cm^2/0.001 abs unit, table 3.2). These observations can be attributed to the low absorptivity of the complex (9737 l/mol/cm) as already indicated before.

The variance of the intercept, S^2_a, calculated was 5.78×10^{-5}. The significance of the intercept was examined using the null hypothesis. The value of t' was obtained as 0.132 whereas the value of t' required for significance is 2.78 at 4 degrees of freedom at the 5% level. Thus, the null hypothesis can be accepted which means that the blank does not interfere in the absorbance measurements. The stability of the complex was ascertained by measurement of absorbances of the solutions, used for

preparing the calibration curve, at regular intervals of time. It was observed that the colour of the complex was retained at its original intensity for an hour. Consequently, it is recommended that the absorbances be measured within an hour of sample preparation and this precaution was followed in all studies involving in this method.

The selectivity of the method for phosphatidyl choline was established by estimating phosphatidyl choline in the presence of the other major constituents of the liposomes viz. cholesterol, cyclosporine, leuprolide acetate, DNA, etc., at the levels at which these materials were included in the liposomes. None of the materials interfered in any way in the estimation of phosphatidyl choline when the Stewart assay was used for the purpose of estimation. Being a widely accepted and used method no need was felt to establish accuracy and precision of the method.

The method was used for estimating phosphatidyl choline from liposomes and from the supernatant obtained in the centrifugation step used for removing the unentrapped drug. A modified version of the Bligh-Dyer two-phase extraction method (New, 1990) was used alone instead of chloroform: methanol mixture, recommended in the reported method, as it was found to give results comparable to these obtained with the original method. Also traces of methanol are reported to interfere with the final partition step in the estimation procedure (Stewart, 1980). As chloroform was liable to form an emulsion with phosphate buffered saline, saturated sodium chloride was added to prevent this. The chloroform layers were passed through a bed of anhydrous sodium sulphate to remove any traces of water. The chloroform extract so obtained was then made up to 10ml and a portion subjected to the procedure detailed above for the estimation of phosphatidyl choline. Mass balance studies revealed good correlation between the amount of phosphatidyl choline added and the amounts recovered from the liposomes and supernatant. Thus the method was found to be satisfactory for estimation of phosphatidyl choline in liposomes.

3.3.2 ESTIMATION OF CHOLESTEROL IN LIPOSOMES

The Zlatkis, Zak and Boyle method (Zlatkis et. al., 1953) was used for estimating cholesterol in liposomes. Here, cholesterol in acetic acid forms a

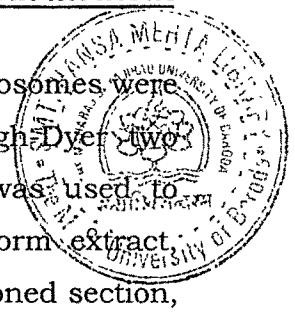
colored complex with ferric chloride and sulphuric acid, which exhibits maximum absorbance at 560nm.

Absorptivity of the complex calculated was 6995l/mol/cm (table 3.4). Linearity of the method was observed from a correlation coefficient of 0.9995 (table 3.3). Beer's law was found to be obeyed between 10 –60 µg/ml (table 3.4 and figure 3.2). Regression analysis of the experimental data was performed and is tabulated along with the experimental data in table 3.3. The regression equation obtained was Y = 0.0177X + 0.0061. The variance of the response variable, S^2_{yx} was calculated as 6.4 x 10^{-5}. This low value illustrated the good fit between the determined and calculated data and thus indicated the low variability of the experiments. This low variability is also reflected in the low value of the standard error of the mean absorbances of the solutions used for obtaining the calibration curve. The variance of the slope, S^2_b was calculated as 3.67 x 10^{-8}. The value of the slope (0.0177, table 3.3) indicates moderate sensitivity of the method. This observation is strengthened by the low values of the absorptivity of the complex mentioned before, the wide range of concentration in which Beer's law is obeyed and the high value of the Sandell's sensitivity coefficient (5.5 x 10^{-5} µg/cm^2/0.001 abs unit, table 3.4). The variance of the intercept, S^2_a, was calculated as 5.5 x 10^{-5}. From the null hypothesis testing, it was seen that the blank does not absorb at the wavelength of interest (calculated t'= 0.8243, tabulated t'= 2.78 at 4 degrees of freedom, at 5% level).

Monitoring of the absorbances of the solutions, used for preparing the calibration curve revealed that the color of the complex was retained as its original intensity for 24h. ANOVA studies of the mean absorbance values of the solutions at different times revealed no significant difference between the readings. Thus, the complex is stable over a period of 24h.

The presence of the other constituents of the liposomes such as phosphatidyl choline, cyclosporine, leuprolide acetate, and DNA etc., at the levels at which these materials were included in the liposomes, did not interfere with the estimation of cholesterol.

Being widely accepted and used method, no need was felt to establish accuracy and precision of the method. Finally the method was used to



estimate cholesterol in liposomes and supernatant when the liposomes were centrifuged to remove unentrapped drug. The modified Bligh-Dyer two phase extraction method, described earlier (section 3.3.1) was used to extract cholesterol from liposomes. A portion of the chloroform extract, obtained by following the procedure detailed in the aforementioned section, was evaporated to dryness by heating on a water bath at 90°C. Care was taken that no chloroform splashed out of the volumetric flask during the process. The contents of the flask were then dissolved in glacial acetic acid before they were subjected to the procedure detailed earlier for the estimation of the cholesterol. There was good agreement between the amount of cholesterol estimated from liposomes and supernatant and that added initially when liposomes were being prepared, indicating the suitability of the method for the estimation of cholesterol from these types of preparations.

Certain precautions need to be taken when the method is being used. If the solutions are shaken prior to measuring the absorbance, upon transferring to cuvettes, air bubbles are seen in the cuvette impending absorbance measurements. The solutions, therefore, need to be transferred carefully to the cuvette, only when they are devoid of air bubbles and that too with no turbulence. Also, the reagents used in the method (concentrated sulphuric acid and glacial acetic acid) are quite corrosive and should be handled carefully.

3.3.3 ESTIMATION OF CYCLOSPORINE IN LIPOSOMES

Cyclosporine in THF/methanol yields a characteristic curve when scanned in the U.V. wavelength range between 200 to 300nm. The scan (figure 3.3) shows absorption maxima at 237.5nm (figure 3.3 and table 3.6). The absorptivity at 238nm (8053 l/mol/cm, Table 3.6) was also found to be satisfactory and hence was selected as the analytical wavelength and used for further investigations. The regression equation was found to be

$Y = 0.0064X + 0.0103$.

A correlation coefficient of 0.999 (table 3.5) indicated that absorbance and concentration of the drug were linearly related. Beer's law was found to be obeyed between 10 - 60 µg/ml (table 3.6). Experimental and calculated

values for the method are presented in table 3.5. The slope of the regressed line (0.0064, table 3.5) indicates low sensitivity of the method as also seen by the value of the Sandell's sensitivity coefficient (1.49×10^{-4}), which, once again, reflects the low absorptivity of the compound.

There was a little variability between the experimental and regressed values. This conclusion was based on the low value of the variance of the response variable, S^2_{yx} (1.7×10^{-5}) and the low values of the standard error of the mean absorbances of the solutions used for preparing the calibration curve. The variance of the slope, S^2_b, was calculated as 1.9×10^{-8} whereas the intercept S^2_a, was determined to be 2.86×10^{-5}. The blank (THF/methanol) does not interfere in the measurements as concluded from t' test of the intercept (calculated t' = 2.64, tabulated t' = 2.78 at 4 degrees of freedom, at 5% level).

The mean absorbance values of the THF/methanol solutions of cyclosporine at different concentrations at preselected time intervals was determined, ANOVA studies of the results indicated no significant difference between the readings. Thus, cyclosporine is stable over a period of 48h in THF /methanol. The presence of the other constituents of the liposomes such as phosphatidyl choline and cholesterol at the levels at which these materials were included in the liposomes, did not interfere with the estimation of cyclosporine.

Accuracy and precision of the above method was not determined since the method is reported earlier. The method was used to estimate the amount of cyclosporine entrapped in liposomes and its concentration in the supernatant, obtained during the recovery of the liposomes by centrifugation. Good mass balance was obtained between the amount of cyclosporine added and that recovered from the liposomes and supernatant, signifying the suitability of the method for this application.

3.3.4 ESTIMATION OF LEUPROLIDE ACETATE IN LIPOSOMES

Leuprolide acetate in 0.1N NaOH yields a characteristic curve when scanned in the U.V. wavelength range between 220 to 400nm. The scan (figure 3.5) shows absorption maxima at 242 and 282nm (table 3.8). Though the absorptivity of the compound was high at 242nm, it was not

selected as the analytical wavelength. The absorptivity at 282nm (6649 l/mol/cm, table 3.8) was also found to be satisfactory and hence was selected as the analytical wavelength and used for further investigations. The regression equation was found to be Y = 0.0053X ϴ.0059.

A correlation coefficient of 0.9995 (table 3.7) indicated that absorbance and concentration of the drug were linearly related. Beer's law was found to be obeyed between 10 - 60 µg/ml (table 3.8). Experimental and calculated values for the method are presented in table 3.7. The slope of the regressed line (0.0053, table 3.7) indicates moderate sensitivity of the method as also seen by the value of the Sandell's sensitivity coefficient (1.9×10^{-4}), which, once again, reflects the moderate absorptivity of the compound.

There was a little variability between the experimental and regressed values. This conclusion was based on the low value of the variance of the response variable, S^2_{yx} (8.5×10^{-5}) and the low values of the standard error of the mean absorbances of the solutions used for preparing the calibration curve. The variance of the slope, S^2_b, was calculated as 9.3×10^{-9} whereas the intercept S^2_a, was determined to be 1.4×10^{-5}. The blank (0.1N NaOH) does not interfere in the measurements as concluded from t'test of the intercept (calculated t' = 2.18, tabulated t' = 2.78 at 4 degrees of freedom, at 5% level).

The mean absorbance values of the aqueous solutions of leuprolide acetate at different concentrations at preselected time intervals was determined, ANOVA studies of the results indicated no significant difference between the readings. Thus, leuprolide acetate is stable over a period of 48h in NaOH. The presence of the other constituents of the liposomes such as phosphatidyl choline and cholesterol at the levels at which these materials were included in the liposomes, did not interfere with the estimation of leuprolide acetate.

Accuracy and precision of the above method was not determined since the method is reported and has been widely used. The method was used to estimate the amount of leuprolide acetate entrapped in liposomes and its concentration in the supernatant, obtained during the recovery of the liposomes by centrifugation. The unentrapped drug was removed from the

liposomal suspension by ficoll- gradient centrifugation (New, 1990). The drug present in the liposomes was estimated by Modified Bligh-Dyer extraction method. Entrapment efficiency was calculated from the difference between the initial amount of leuprolide acetate added and that presents in the unentrapped form and was expressed as % of total amount of leuprolide acetate added. Good mass balance was obtained between the amount of leuprolide acetate added and that recovered from the liposomes and supernatant, signifying the suitability of the method for this application.

3.3.5 ESTIMATION OF DNA IN LIPOSOMES

DNA in Tris buffer yields a characteristic curve when scanned in the U.V. wavelength range between 200 to 400nm. The scan (figure 3.7) shows absorption maxima at 260nm (table 3.10). The regression equation was found to be Y = 0.0355X + 0.015.

A correlation coefficient of 0.9998 (table 3.9) indicated that absorbance and concentration of the drug were linearly related. Beer's law was found to be obeyed between 4 - 22 µg/ml (table 3.10). Experimental and calculated values for the method are presented in table 3.9. The slope of the regressed line (0.0355, table 3.9) indicates moderate sensitivity of the method. There was a little variability between the experimental and regressed values. This conclusion was based on the low value of the variance of the response variable, S^2_{yx} (2.6×10^{-4}) and the low values of the standard error of the mean absorbances of the solutions used for preparing the calibration curve. The variance of the slope, S^2_b, was calculated as 1.70×10^{-7} whereas the intercept S^2_a, was determined to be 1.56×10^{-4}. The blank (tris buffer) does not interfere in the measurements as concluded fromt'test of the intercept (calculatedt'= 0.48, tabulatedt'= 2.78 at 4 degrees of freedom, at 5% level). The presence of the other constituents of the liposomes such as phosphatidyl choline and cholesterol at the levels at which these materials were included in the liposomes, did not interfere with the estimation of DNA.

The method was used to estimate the amount of DNA entrapped in liposomes and its concentration in the supernatant, obtained during the recovery of the liposomes by centrifugation. The unentrapped DNA was

removed from the liposomal suspension by ficoll- gradient centrifugation (New, 1990). The DNA present in the liposomes was estimated by Modified Bligh-Dyer extraction method. Entrapment efficiency was calculated from the difference between the initial amount of leuprolide acetate added and that presents in the unentrapped form and was expressed as % of total amount of DNA added. Good mass balance was obtained between the amount of DNA added and that recovered from the liposomes and supernatant, signifying the suitability of the method for this application.

3.3.6 ESTIMATION OF LEUPROLIDE ACETATE IN PHOSPHATE BUFFER SALINE, pH 7.4 (PBS)

Leuprolide acetate in phosphate buffered saline, pH 7.4 (PBS) yields a characteristic curve when scanned in the UV wavelength range between 220nm to 400nm with the scan (figure 3.9) showing maxima at 280nm. The absorptivity at 280nm (4593 l/mol/cm, table 3.12) was also found to be satisfactory and hence was selected as the analytical wavelength and used for further investigations. The regression equation was found to be
$Y = 0.0034x + 0.0119$.

A correlation coefficient of 0.9971 (table 3.11) indicated that the absorbance and concentration of the drug were linearly related. Beer's law was found to be obeyed between 10 - 60 µg/ml (table 3.12). The variance of the response variable, S^2_{yx} was calculated as 1.68×10^{-5}. This low value illustrated the good fit between the determined and calculated data and thus indicated the low variability of the experiments. This low variability is also reflected in the low value of the standard error of the mean absorbances of the solutions used for obtaining the calibration curve. The variance of the slope, S^2_b was calculated as 9.68×10^{-8}. The value of the slope (0.0034, table 3.11) indicates low sensitivity of the method and supported by the low value of the Sandell's sensitivity coefficient (1.38×10^{-4} µg/cm^2/0.001 abs unit, table 3.12). The variance of the intercept, S^2_a, was calculated as 2.45×10^{-5}. The intercept was subjected to t' test for significance and was not found to be significantly different from zero (calculated t' = 2.4, tabulated t' = 2.78 at 4 degrees of freedom, at 5% level). This means that the blank (PBS) does not interfere in the absorbance measurements.

The mean absorbance values of the leuprolide acetate solution in PBS at different concentrations at preselected time intervals was determined, ANOVA studies of the results indicated no significant difference between the readings. Thus, leuprolide acetate is stable over a period of 48h in PBS.

The presence of the other constituents of the liposomes such as phosphatidyl choline and cholesterol at the levels at which these materials were included in the liposomes, did not interfere with the estimation of leuprolide acetate.

The method was used to obtain the release profile of leuprolide acetate from conventional and sterically stabilized liposomes *in vitro* using an appropriate set up. The reproducible drug release profiles obtained indicated that the developed method was suitable for the desired application.

3.3.7 ESTIMATION OF DNA IN PHOSPHATE BUFFER SALINE, pH 7.4 (PBS)

DNA in phosphate buffered saline pH 7.4 (PBS) yields a characteristic curve when scanned in the U.V. wavelength range between 200 to 400nm. The scan (figure 3.11) shows absorption maxima at 260nm, which was selected as the analytical wavelength for measuring the DNA in PBS. The regression equation was found to be $Y = 0.023x - 0.0063$.

A correlation coefficient of 0.9995 (table 3.13) indicated that absorbance and concentration of the drug were linearly related. Beer's law was found to be obeyed between 4 - 22 µg/ml (table 3.13 and figure 3.12). Experimental and calculated values for the method are presented in Table 3.14. The slope of the regressed line (0.023, table 3.14) indicates moderate sensitivity of the method. There was a little variability between the experimental and regressed values. This conclusion was based on the low value of the variance of the response variable, S^2_{yx} (1.5×10^{-5}) and the low values of the standard error of the mean absorbances of the solutions used for preparing the calibration curve. The variance of the slope, S^2_b, was calculated as 8.8×10^{-9} whereas the intercept S^2_a, was determined to be 1.3×10^{-5}. The blank does not interfere in the measurements as concluded from t' test of the

intercept (calculated t'= 1.72, tabulated t'= 2.78 at 4 degrees of freedom, at 5% level).

The mean absorbance values of the DNA solution in PBS at different concentrations at preselected time intervals was determined, ANOVA studies of the results indicated no significant difference between the readings. Thus, DNA is stable over a period of 48h in PBS.

The presence of the other constituents of the liposomes such as phosphatidyl choline and cholesterol at the levels at which these materials were included in the liposomes, did not interfere with the estimation of DNA The method was used to obtain the release profile of DNA from conventional and sterically stabilized liposomes *in vitro* using an appropriate set up. The reproducible release profiles obtained indicated that the developed method was suitable for the desired application.

3.3.8 ESTIMATION OF POLYETHYLENE GLYCOL DERIVATIVES IN LIPOSOMES

This assay method for the estimation of polyethylene glycol is based on complex formation between picrates and polyethylene glycols in dichloromethane. The complex in dichloromethane exhibits maximum absorbance at 378nm.

3.3.8.1 Estimation of methoxy polyethylene glycol 5000 activated with cyanuric chloride- phosphatidyl ethanolamine conjugate (MPEG5000-CC-PE)

Absorptivity of the complex was calculated as 81986 l/mol/cm (table 3.16). A correlation coefficient of 0.9995 (table 3.15) indicated a linear relationship between absorbance and concentration of mPEG5000-CC-PE taken for complex formation. Beer's law was found to be obeyed between 10 –60 µg/ml (table 3.16). The data of regression analysis on the collected data along with the raw data are presented in Table 3.15. The regression equation obtained was Y = 0.0136X + 0.0293.

The variance of the response variable, S^2_{yx} was obtained as 1.4×10^{-4}. This low value denoted the closeness of experimental points to the least squares line and thus, the low variability in the procedure. This low variability can also be seen from the low value of the standard error of the mean

absorbances of the solutions used for obtaining the calibration curve (table 3.15). The variance of the slope, S^2_b was calculated as 8.1×10^{-8}. The value of the slope (0.0136, Table 3.16) indicated moderate sensitivity of the method which is well supported by the value of apparent molar absorptivity of the compound at 378nm, a fact supported by the value of the Sandells sensitivity coefficient (7.05×10^{-5} µg/cm^2/0.001 abs unit, table 3.16).

The variance of the intercept, S^2_a, calculated was 1.22×10^{-4}. The null hypothesis was used to determine whether the intercept was significantly different from zero. The value of t'was obtained as 2.64 whereas the value of t'required for significance is 2.78 at 4 degrees of freedom at the 5% level. Thus, the null hypothesis can be accepted which means that the blank does not interfere in the absorbance measurements. The stability of the complex was ascertained by measurement of absorbances of the solutions, used for preparing the calibration curve, at regular intervals of time. It was observed that the colour of the complex was retained at its original intensity for an hour. Consequently, it is recommended that the absorbances be measured within an hour of sample preparation and this precaution was followed in all studies involving in this method.

The selectivity of the method for mPEG5000-CC-PE was established by estimating in the presence of the other major constituents of the liposomes viz. cholesterol, phosphatidyl choline, leuprolide acetate and DNA at the levels at which these materials were included in the liposomes. None of the materials interfered in any way in the estimation of mPEG5000-CC-PE.

The method was used for estimating mPEG5000-CC-PE from liposomes and from the supernatant obtained in the centrifugation step used for removing the unentrapped drug. Mass balance studies revealed good correlation between the amount of mPEG5000-CC-PE added and the amounts recovered from the liposomes and supernatant. Thus the method was found to be satisfactory for estimation of mPEG5000-CC-PE in liposomes.

3.3.8.2 Estimation of methoxy polyethylene glycol 2000 activated with cyanuric chloride-phosphatidyl ethanolamine conjugate (MPEG2000-CC-PE)

Absorptivity of the complex was calculated as 38315 l/mol/cm (table 3.17). A correlation coefficient of 0.9988 (table 3.17) indicated a linear relationship between absorbance and concentration of mPEG2000-CC-PE taken for complex formation. Beer's law was found to be obeyed between 10 –60 µg/ml (table 3.18). The data of regression analysis on the collected data along with the raw data are presented in table 3.17. The regression equation obtained was Y = 0.0134X + 0.0103.

The variance of the response variable, S^2_{yx} was obtained as 6.5 x 10^{-5}. This low value denoted the closeness of experimental points to the least squares line and thus, the low variability in the procedure. This low variability can also be seen from the low value of the standard error of the mean absorbances of the solutions used for obtaining the calibration curve (table 3.17). The variance of the slope, S^2_b was calculated as 5.3 x 10^{-8}. The value of the slope (0.0134, table 3.17) indicated moderate sensitivity of the method which is well supported by the value of apparent molar absorptivity of the compound at 378nm, a fact supported by the value of the Sandell's sensitivity coefficient (7.3 x 10^{-5} µg/cm^2/0.001 abs unit, table 3.18).

The variance of the intercept, S^2_a, calculated was 5.6 x 10^{-5}. The significance of the intercept was examined using the null hypothesis. The value of t' was obtained as 1.37 whereas the value of t' required for significance is 2.78 at 4 degrees of freedom at the 5% level. Thus, the null hypothesis can be accepted which means that the blank does not interfere in the absorbance measurements. The stability of the complex was ascertained by measurement of absorbances of the solutions, used for preparing the calibration curve, at regular intervals of time. It was observed that the colour of the complex was retained at its original intensity for an hour. The selectivity of the method for mPEG2000-CC-PE was established by estimating in the presence of the other major constituents of the liposomes viz. cholesterol, phosphatidyl choline, leuprolide acetate and DNA at the levels at which these materials were included in the liposomes. None

of the materials interfered in any way in the estimation of mPEG2000-CC-PE.

The method was used for estimating mPEG2000-CC-PE from liposomes and from the supernatant obtained in the centrifugation step used for removing the unentrapped drug. Mass balance studies revealed good correlation between the amount of mPEG2000-CC-PE added and the amounts recovered from the liposomes and supernatant. Thus the method was found to be satisfactory for estimation of mPEG2000-CC-PE in liposomes.

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