# Chapter 4
# **Analytical Methods**

# 4. ANALYTICAL METHODS

## 4.1 Introduction

In addition to possess the desired characteristics of accuracy, precision, reproducibility, ruggedness, robustness etc., the analytical methods used to quantify drug in liposomes should possess the ability to be used in conjunction with techniques common to liposomal adjuvants. Techniques are adopted to ensure minimum interference from the other components of the formulation. The methods used should be stability indicating which would, when used, draw attention to any potential incompatibility between the various components of the liposomes. The UV-Visible method of estimation was developed based on the observation that Paclitaxel (PCL) and Irinotecan Hydrochloride (IH) showed strong absorbance in the UV region of the electromagnetic spectrum in solvents like methanol: chloroform, pH 7.4 Phosphate Buffer (pH 7.4 PB) with 20 % methanol.

## 4.2 Estimation of Paclitaxel:

*Solutions:*

Stock solution of Paclitaxel (100 μg/ml) was prepared by dissolving 10 mg of Paclitaxel in 100 ml methanol: Chloroform (9:1 ratio).

Stock solution of Paclitaxel (100 μg/ml) was prepared by dissolving 10 mg of Paclitaxel in 100 ml methanol.

Phosphate buffered saline pH 7.4 (PBS) and PBS pH 5.0 was prepared as per the procedure given in the Indian Pharmacopoeia (1996)

*Procedure for calibration plot:*

Stock solution of Paclitaxel in the solvent system in which the calibration plot is to be prepared (Methanol: Chloroform (9:1 ratio) mixture, pH 7.4 PBS and PBS pH 5.0 with 20 % methanol) was prepared by dissolving 10 mg of PCL in 100 ml of the solvent. Suitable aliquots of the 100 μg/ml stock solution of PCL were pipetted into 10ml

volumetric flasks and volume was made upto 10 ml using the same solvent to give final concentration of 5, 10, 15, 20, 25 and 30 μg/ml. The solutions were shaken well and their absorbances were measured at 228 nm using suitable blank on a Shimadzu 1601 UV-Visible spectrophotometer. The above procedure was repeated six times and the mean absorbance value was determined. The absorbance values obtained in Methanol: Chloroform (9:1 ratio) mixture, pH 7.4 and PBS pH 5.0 with 20 % methanol are tabulated in Table 4.1 to Table 4.3 respectively.

*Stability and selectivity:*

Stability of the solutions of Paclitaxel in methanol: chloroform (9:1 ratio) mixture, pH 5.0 and pH 7.4 PBS with 20 % methanol, was studied by preparing the calibration curve at 228 nm and 229 nm over a period of 72h at room temperature. The above method for estimating Paclitaxel was carried out in the presence of different components of liposomal system to ascertain the selectivity of the method and also to check the absence of interference of the lipid mixture in the absorptivity of PCL.

*Accuracy and Precision:*

In order to determine the accuracy and precision of the developed method, known amounts of PCL (10 μg/ml, 20 μg/ml and 25 μg/ml) were subjected to recovery studies as per the procedure described above. The results obtained are tabulated in Table 4.4 to Table 4.6 respectively for methanol: chloroform (9:1 ratio) mixture and pH 5.0 and pH 7.4 PBS with 20 % methanol.

*Estimation of Paclitaxel from liposomes:*

To 0.1 ml of liposome in a 10 ml volumetric flask, Methanol: Chloroform (9:1 ratio) was added to break the liposome and the volume was made up with methanol. The absorbance was measured at 229nm against a blank comprising of empty liposomes diluted with methanol: Chloroform (9:1 ratio) in the similar manner. Triplicate estimations were made and the mean absorbances were determined. The amount of Paclitaxel in the liposomes or supernatant was then obtained using the regression equation.

**Table 4.1. Calibration curve for Paclitaxel in methanol: chloroform (9:1 ratio)**

| Concentration (µg/ml) | Mean absorbance* ± S. D. |
|---|---|
| 1 | 0.060 ± 0.0064 |
| 3 | 0.119 ± 0.0019 |
| 5 | 0.179 ± 0.0019 |
| 10 | 0.336 ± 0.0012 |
| 15 | 0.490 ± 0.0018 |
| 20 | 0.670 ± 0.0020 |
| 25 | 0.805 ± 0.0014 |
| 30 | 0.986 ± 0.0022 |

Regression equation: $y = 0.0318x + 0.0219$
Correlation Co-efficient $R^2 = 0.9993$
*Mean of 6 values.

**Table 4.2. Calibration curve for Paclitaxel in PBS pH 7.4 (with 20% methanol)**

| Concentration (µg/ml) | Mean absorbance* ± S. D. |
|---|---|
| 1 | 0.037 ± 0.0010 |
| 2 | 0.072 ± 0.0014 |
| 4 | 0.151 ± 0.0008 |
| 6 | 0.234 ± 0.0014 |
| 8 | 0.330 ± 0.0016 |
| 10 | 0.399 ± 0.0012 |
| 12 | 0.488 ± 0.0018 |
| 14 | 0.580 ± 0.0016 |
| 16 | 0.665 ± 0.0012 |
| 18 | 0.752 ± 0.0006 |
| 20 | 0.827 ± 0.0008 |

Regression equation: $y = 0.0422x - 0.0132$
Correlation Co-efficient $R^2 = 0.9996$
*Mean of 6 values.

**Table 4.3. Calibration curve for Paclitaxel in PBS pH 5.0 (with 20% methanol)**

| Concentration (µg/ml) | Mean absorbance* ± S. D. |
|---|---|
| 1 | 0.035 ± 0.0013 |
| 2 | 0.074 ± 0.0018 |
| 4 | 0.149 ± 0.0023 |
| 6 | 0.230 ± 0.0046 |
| 8 | 0.327 ± 0.0017 |
| 10 | 0.394 ± 0.0038 |
| 12 | 0.480 ± 0.0076 |
| 14 | 0.575 ± 0.0028 |
| 16 | 0.661 ± 0.0043 |
| 18 | 0.749 ± 0.0062 |
| 20 | 0.819 ± 0.0075 |

Regression equation: $y = 0.0419x - 0.0141$
Correlation Co-efficient $R^2 = 0.9993$
*Mean of 6 values.

**Table 4.4. Evaluation of Accuracy and precision of the estimation method of Paclitaxel in methanol: chloroform (9:1 ratio) mixture.**

| Theoretical concentration of PCL (µg/ml) | Determined value (µg/ml) | Coefficient of variance (CV) | Standard error | Confidence limits* |
|---|---|---|---|---|
| 10 | 10.07 | 0.357 | 0.00049 | 10.07 ± 0.0013 |
| 20 | 20.05 | 0.299 | 0.00082 | 20.05 ± 0.0021 |
| 25 | 25.16 | 0.174 | 0.00057 | 25.16 ± 0.0015 |

* At 95% confidence level; $t_{tab} = 2.571$ for 5 degrees of freedom

**Table 4.5. Evaluation of Accuracy and precision of the estimation method of Paclitaxel in pH 7.4 PBS with 20 % methanol**

| Theoretical concentration of PCL (µg/ml) | Determined value (µg/ml) | Coefficient of variance (CV) | Standard error | Confidence limits* |
|---|---|---|---|---|
| 10 | 10.03 | 0.3007 | 0.00049 | 10.03 ± 0.0013 |
| 16 | 15.95 | 0.1805 | 0.00049 | 15.95 ± 0.0013 |
| 20 | 20.10 | 0.0967 | 0.00033 | 20.10 ± 0.0008 |

* At 95% confidence level; $t_{tab} = 2.571$ for 5 degrees of freedom

**Table 4.6. Evaluation of Accuracy and precision of the estimation method of Paclitaxel in pH 5.0 PBS with 20 % methanol.**

| Theoretical concentration of PCL (µg/ml) | Determined value (µg/ml) | Coefficient of variance (CV) | Standard error | Confidence limits* |
|---|---|---|---|---|
| 10 | 10.03 | 0.9645 | 0.0016 | 10.03 ± 0.0040 |
| 16 | 15.95 | 0.6505 | 0.0018 | 15.95 ± 0.0045 |
| 20 | 20.10 | 0. 9158 | 0.0031 | 20.10 ± 0.0079 |

* At 95% confidence level; $t_{tab} = 2.571$ for 5 degrees of freedom

## High Performance Liquid Chromatography

The amount of PCL in aqueous phase was analyzed by HPLC (Dionex) following experimental conditions: Hypersil BDS $C_{18}$ (250 x 4.6 mm i.d., 5µ); Mobile phase Acetonitrile: water (65:35 v/v); flow rate 1ml/min; temperature, ambient and measured wavelength 227nm for PCL (Elkharraz *et. al.*, 2006: Potineni *et. al.*, 2003: Kim. *et. al.*, 2005: Xie *et. al.*, 2007; Crosasso *et. al.*, 2000).

## Estimation of PCL

### *Preparation of stock solution*

50 mg of PCL was taken into a 50ml volumetric flask. To this acetonitrile: water mixture (65:35 v/v) added and vortexed to get a clear solution and finally made up the volume to 50 ml with same solvent system.

### *Preparation of working standard solution*

From the stock solution, standard solutions were prepared to contain 0.1, 0.25, 0.5, 1, 2, 5, and 10 µg/ml of PCL using mobile phase (ACN: water 65:35, v/v). The calibration curves were drawn by plotting the peak area versus concentration of PCL and are shown in Figure 4.1. This standard curve was linearly regressed and statistical parameters related to it were derived (Table 4.7)

**Table 4.7. Calibration plot of PCL standard solutions**

| Concentration (µg/ml) | Peak area for PCL* (mVA) |
|---|---|
| 0.10 | 0.0306 ± 0.0012 |
| 0.25 | 0.0752 ± 0.0011 |
| 0.50 | 0.1928 ± 0.0018 |
| 1 | 0.4882 ± 0.0024 |
| 2 | 1.1198 ± 0.0045 |
| 5 | 3.3618 ± 0.0086 |
| 10 | 6.7726 ± 0.0084 |

Regression equation: y = 0.6907x - 0.1399
Correlation Co-efficient: $R^2$= 0.9991
*Mean of 6 values.

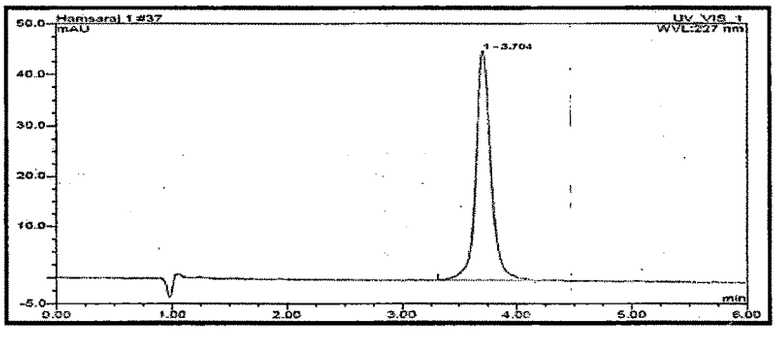


**Figure 4.1. HPLC histogram for PCL.**

**Table 4.8. Evaluation of Accuracy and precision of the estimation method of Paclitaxel in by HPLC method.**

| Theoretical concentration of PCL (µg/ml) | Determined value (µg/ml) | Coefficient of variance (CV) | Standard error | Confidence limits* |
|---|---|---|---|---|
| 0.25 | 0.251 | 1.463 | 0.00045 | 0.251 ± 0.0012 |
| 1.0 | 0.992 | 0.492 | 0.00098 | 0.992 ± 0.0025 |
| 5.0 | 5.021 | 0.256 | 0.00351 | 5.021 ± 0.0090 |

* At 95% confidence level; $t_{tab}$ = 2.571 for 5 degrees of freedom

## 4.4 Estimation of Irinotecan Hydrochloride:

The UV-Visible method of estimation was developed based on the observation that Irinotecan Hydrochloride (IH) showed strong absorbance in the UV region of the electromagnetic spectrum in solvents like double distilled water, methanol: chloroform mixture, Phosphate Buffer (pH 5.0 and 7.4 PBS).

***Solutions:***

Stock solution of Irinotecan Hydrochloride (100 µg/ml) was prepared by dissolving 10 mg of Irinotecan Hydrochloride in 100 ml double distilled water.

Stock solution of Irinotecan Hydrochloride (100 µg/ml) was prepared by dissolving 10 mg of Irinotecan Hydrochloride in 100 ml methanol: Chloroform (9:1 ratio).

Stock solution of Irinotecan Hydrochloride (100 µg/ml) was prepared by dissolving 10 mg of Irinotecan Hydrochloride in 100 ml pH 5.0 & 7.4 PBS.

***Procedure for calibration curve:***

Stock solution of Irinotecan Hydrochloride in the solvent system in which the calibration plot is to be prepared (Double distilled water, Methanol: Chloroform (9:1 ratio) mixture, pH 5.0 & 7.4 PBS) was prepared by dissolving 10 mg of IH in 100 ml of the solvent. Suitable aliquots of the 100 µg/ml stock solution of IH were pipetted into 10ml volumetric flasks and volume was made upto 10 ml using the same solvent to give final concentration of 1, 2, 3, 4, 5, 10, 15 and 20 µg/ml. The solutions were shaken well and their absorbances were measured at 256nm and 357nm using suitable blank on a Shimadzu 1601 UV-Visible spectrophotometer. The above procedure was repeated six times and the mean absorbance value was determined. The absorbance values obtained in double distilled water, Methanol: Chloroform (9:1 ratio) mixture, pH 5.0 and 7.4 PBS are tabulated in Table 4.9 to Table 4.12 respectively.

***Stability and selectivity:***

Stability of the solutions of Irinotecan Hydrochloride in double distilled water, Methanol: Chloroform (9:1 ratio) mixture, pH 5.0 and pH 7.4 PBS used for preparing the calibration curve was ascertained by observing the changes in their absorbance at the analytical wavelength, over a period of 72 hours.

The above method for estimating Irinotecan Hydrochloride was carried out in the presence of different components of liposomal system to ascertain the selectivity of the method and also to check the absence of interference of the lipid mixture in the absorptivity of IH.

***Accuracy and Precision:***

In order to determine the accuracy and precision of the developed method, known amounts of IH (10 µg/ml, 15 µg/ml and 20 µg/ml) were subjected to recovery studies as per the procedure described above. The results obtained are tabulated in Table 4.13 to Table 4.16 respectively for double distilled water, methanol: chloroform (9:1 ratio) mixture and pH 5.0 and pH 7.4 PBS.

**Estimation of Irinotecan Hydrochloride from liposomes:**

To 0.1 ml of liposome was diluted to 1ml in an effendorf tube and centrifuged at 25,000 rpm to settle liposomes for 30 min. Then the supernatant was taken in a 10 ml volumetric flask, double distilled water was added to made up the volume. The absorbance was measured at 361nm against a blank comprising of double distilled water in the similar manner. Triplicate estimations were made and the mean absorbances were determined. The amount of unentrapped Irinotecan Hydrochloride in the supernatant was then obtained using the regression equation.

**Table 4.9. Calibration curve for Irinotecan Hydrochloride in double distilled water**

| **Concentration** (μg/ml) | **Mean absorbance* ± S. D.** |
|---|---|
| 1 | 0.039 ± 0.0016 |
| 2 | 0.077 ± 0.0023 |
| 3 | 0.134 ± 0.0010 |
| 4 | 0.186 ± 0.0019 |
| 5 | 0.233 ± 0.0015 |
| 10 | 0.460 ± 0.0026 |
| 15 | 0.722 ± 0.0008 |
| 20 | 0.973 ± 0.0024 |

Regression equation: $y = 0.0497 x + 0.0182$
Correlation Co-efficient $R^2 = 0.9996$
*Mean of 6 values.

**Table 4.10. Calibration curve for Irinotecan Hydrochloride in methanol: chloroform (9:1 ratio)**

| **Concentration** (μg/ml) | **Mean absorbance* ± S. D.** |
|---|---|
| 1 | 0.100 ± 0.0032 |
| 2 | 0.170 ± 0.0021 |
| 3 | 0.250 ± 0.0020 |
| 4 | 0.334 ± 0.0016 |
| 5 | 0.415 ± 0.0012 |
| 10 | 0.828 ± 0.0022 |

Regression equation: $y = 0.0807 x + 0.0119$
Correlation Co-efficient $R^2 = 0.9998$
*Mean of 6 values.

**Table 4.11. Calibration curve for Irinotecan Hydrochloride in PBS pH 7.4**

| **Concentration** (µg/ml) | **Mean absorbance* ± S. D.** |
|---|---|
| 1 | 0.045 ± 0.005 |
| 2 | 0.097 ± 0.010 |
| 3 | 0.149 ± 0.008 |
| 4 | 0.201 ± 0.012 |
| 5 | 0.275 ± 0.016 |
| 10 | 0.591 ± 0.021 |
| 15 | 0.924 ± 0.017 |
| 20 | 1.236 ± 0.028 |

Regression equation: $y = 0.0635 x - 0.0368$
Correlation Co-efficient $R^2 = 0.9994$
*Mean of 6 values.

**Table 4.12. Calibration curve for Irinotecan Hydrochloride in PBS pH 5.0**

| **Concentration** (µg/ml) | **Mean absorbance* ± S. D.** |
|---|---|
| 1 | 0.041 ± 0.008 |
| 2 | 0.093 ± 0.014 |
| 3 | 0.147 ± 0.011 |
| 4 | 0.197 ± 0.026 |
| 5 | 0.262 ± 0.017 |
| 10 | 0.561 ± 0.028 |
| 15 | 0.856 ± 0.031 |
| 20 | 1.124 ± 0.023 |

Regression equation: $y = 0.0578 x - 0.0237$
Correlation Co-efficient $R^2 = 0.9996$
*Mean of 6 values.

**Table 4.13. Evaluation of Accuracy and precision of the estimation method of Irinotecan Hydrochloride in double distilled water.**

| Theoretical concentration of IH (µg/ml) | Determined value (µg/ml) | Coefficient of variance (CV) | Standard error | Confidence limits* |
|---|---|---|---|---|
| 10 | 10.14 | 0.5652 | 0.0011 | 10.14 ± 0.0027 |
| 15 | 15.09 | 0.1108 | 0.0003 | 15.09 ± 0.0008 |
| 20 | 20.18 | 0.2467 | 0.0010 | 20.18 ± 0.0025 |

* At 95% confidence level; $t_{tab} = 2.571$ for 5 degrees of freedom.

**Table 4.14. Evaluation of Accuracy and precision of the estimation method of Irinotecan Hydrochloride in methanol: chloroform (9:1 ratio) mixture.**

| Theoretical concentration of IH (µg/ml) | Determined value (µg/ml) | Coefficient of variance (CV) | Standard error | Confidence limits* |
|---|---|---|---|---|
| 4 | 4.03 | 0.8602 | 0.00065 | 4.03 ± 0.0017 |
| 5 | 5.10 | 0.5150 | 0.00048 | 5.10 ± 0.0013 |
| 10 | 10.06 | 0.4783 | 0.00089 | 10.06 ± 0.0023 |

* At 95% confidence level; $t_{tab} = 2.571$ for 5 degrees of freedom

**Table 4.15. Evaluation of Accuracy and precision of the estimation method of Irinotecan Hydrochloride in pH 7.4 PBS.**

| Theoretical concentration of IH (µg/ml) | Determined value (µg/ml) | Coefficient of variance (CV) | Standard error | Confidence limits* |
|---|---|---|---|---|
| 10 | 10.04 | 0.3553 | 0.0009 | 10.04 ± 0.0022 |
| 15 | 14.97 | 0.1840 | 0.0007 | 14.97 ± 0.0018 |
| 20 | 20.11 | 0.2265 | 0.0011 | 20.11 ± 0.0029 |

* At 95% confidence level; $t_{tab} = 2.571$ for 5 degrees of freedom

**Table 4.16. Evaluation of Accuracy and precision of the estimation method of Irinotecan Hydrochloride in pH 5.0 PBS.**

| Theoretical concentration of IH (µg/ml) | Determined value (µg/ml) | Coefficient of variance (CV) | Standard error | Confidence limits* |
|---|---|---|---|---|
| 10 | 10.04 | 3.2085 | 0.0074 | 10.04 ± 0.0189 |
| 15 | 14.97 | 2.2313 | 0.0078 | 14.97 ± 0.0200 |
| 20 | 20.11 | 1.7794 | 0.0082 | 20.11 ± 0.0210 |

* At 95% confidence level; $t_{tab} = 2.571$ for 5 degrees of freedom

## 4.5 Fluorimetric estimation of Irinotecan Hydrochloride in double distilled water and human serum and human plasma

### Instrumentation

All Fluorimetric estimations were performed on a Shimadzu RF-540 spectrofluorometer (Shimadzu Corporation, Japan) equipped with a xenon lamp. The various experimental conditions like the slit width for excitation (kept at 3) and emission (kept at 5) and the excitation and emission wavelengths ($\lambda_{excitation}$ = 374 nm; $\lambda_{emission}$ = 435 nm) were optimized (Sadzuka et. al., 1999).

### Preparation of calibration plot in double distilled water

Stock solution of Irinotecan Hydrochloride in Double distilled water was prepared by dissolving 10 mg of IH in 100 ml of the solvent. Suitable aliquots of the 100 µg/ml stock solution of IH were pipetted into 10ml volumetric flasks and volume was made upto 10 ml using the same solvent to give required final concentration. The solutions were shaken well and the relative fluorescence intensity was measured setting the $\lambda_{excitation}$ at 374 nm and the corresponding $\lambda_{emission}$ peak intensity was measured at 435 ± 2nm (slit widths as mentioned above) using a Shimadzu RF-540 spectrofluorometer (Shimadzu Corporation, Japan) against suitable blank. The above procedure was repeated six times and the mean relative fluorescence intensity values were determined. The mean relative fluorescence intensity values obtained in double distilled water are tabulated in Table 4.17. The $\lambda_{emission}$ peaks of IH in double distilled water are shown in Figure 4.2.

### Construction of calibration plot in human serum and plasma

To 0.1 ml of human serum or plasma required quantity of drug solution was added (from a stock solution of IH in double distilled water) to obtain the final concentration of IH ranging between 1 to 100ng/ml. The contents were gently mixed to ensure uniform mixing and kept aside for few minutes away from light. To the samples was added 2.0ml of methanol or 1ml 5% trichloroacetic acid solution, mixed for 5 min followed by centrifugation at 1500 x g for 10 min. The supernatant (organic layer) was transferred into the glass tubes. The extraction procedure was repeated twice and from the combined

organic layer 0.2ml was taken and diluted to 10 ml with double distilled water. The fluorescence of extracted drug was measured in Shimadzu RF-540 spectrofluorometer (Shimadzu Corporation, Japan) at wavelength of excitation at 374 nm and emission at 435 ± 2nm. All the estimations were carried out between 22 °C - 27 °C, and care was taken to prevent light exposure and solvent evaporation at every stage of estimation. Calibration plots were constructed for the measured relative fluorescence intensity against drug concentration (Table 4.19). Accuracy and precision of the method was determined by performing recovery studies after addition of IH in human serum and plasma in triplicate.

***Accuracy and Precision***

In order to determine the accuracy and precision of the developed method, known amounts of IH (10 ng/ml, 50 ng/ml and 100 ng/ml) were subjected to recovery studies as per the procedure described earlier. The results obtained are tabulated in Table 4.18 and Table 4.20 respectively for human serum and plasma.

**Table 4.17. Calibration curve for Irinotecan Hydrochloride in double distilled water**

| **Concentration** (ng/ml) | **Mean relative fluorescence intensity ± S. D.*** |
|---|---|
| 1 | 0.68 ± 0.08 |
| 5 | 3.38 ± 0.10 |
| 10 | 6.80 ± 0.14 |
| 25 | 12.90 ± 0.27 |
| 50 | 18.60 ± 0.24 |
| 75 | 29.60 ± 0.33 |
| 100 | 32.50 ± 0.48 |

Regression equation: $y = 2.8293x + 4.2123$
Correlation Co-efficient $R^2 = 0.9996$
*Mean of 6 values.

**Table 4.18. Evaluation of Accuracy and precision of the estimation method of Irinotecan Hydrochloride in double distilled water ($\lambda_{excitation}$ at 374 nm; $\lambda_{emission}$ at 435 ± 2 nm)**

| Theoretical concentration of IH (µg/ml) | Determined value (µg/ml) | Coefficient of variance (CV) | Standard error | Confidence limits* |
|---|---|---|---|---|
| 10 | 10.14 | 2.0588 | 0.05714 | 10.14 ± 0.1469 |
| 50 | 50.49 | 1.2903 | 0.09796 | 50.49 ± 0.2519 |
| 100 | 102.18 | 1.4769 | 0.19592 | 102.18 ± 0.5037 |

* At 95% confidence level; $t_{tab}$ = 2.571 for 5 degrees of freedom

**Table 4.19. Calibration curve for Irinotecan Hydrochloride in Human serum/plasma**

| Concentration (ng/ml) | Mean relative fluorescence intensity ± S. D.* |
|---|---|
| 1 | 9.68 ± 0.18 |
| 2 | 18.08 ± 0.20 |
| 3 | 26.90 ± 0.12 |
| 4 | 41.40 ± 0.37 |
| 5 | 57.10 ± 0.44 |
| 10 | 104.3 ± 0.63 |

Regression equation: y = 15.1x + 18.6
Correlation Co-efficient $R^2$= 0.9995
*Mean of 6 values.

**Table 4.20. Evaluation of Accuracy and precision of the estimation method of Irinotecan Hydrochloride in double distilled water ($\lambda_{excitation}$ at 374 nm; $\lambda_{emission}$ at 435 ± 2 nm)**

| Theoretical concentration of IH (µg/ml) | Determined value (µg/ml) | Coefficient of variance (CV) | Standard error | Confidence limits* |
|---|---|---|---|---|
| 1 | 1.04 | 0.1859 | 0.0735 | 1.04 ± 0.1889 |
| 5 | 5.29 | 0.7706 | 0.1796 | 5.29 ± 0.4617 |
| 10 | 10.19 | 0.6040 | 0.2571 | 10.19 ± 0.6611 |

* At 95% confidence level; $t_{tab}$ = 2.571 for 5 degrees of freedom.

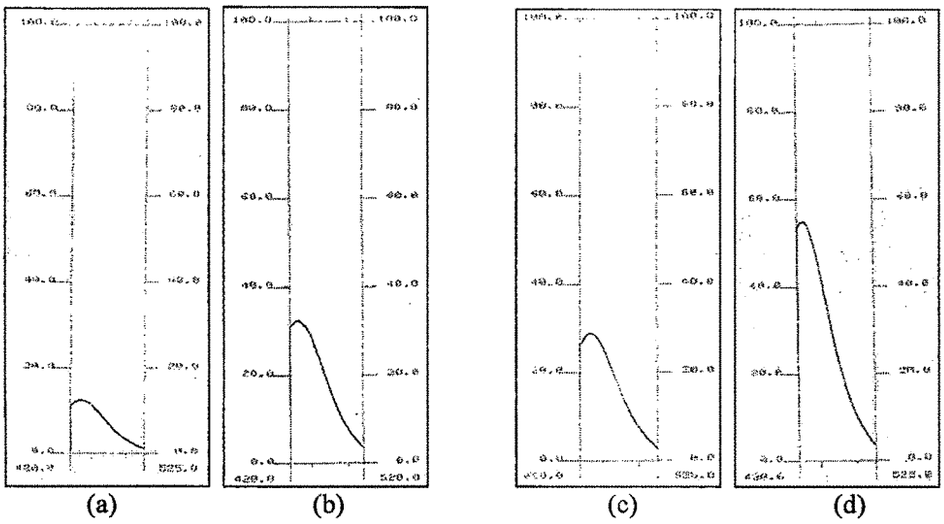


**Figure 4.2. $\lambda_{emission}$ peaks at 435 ± 2 nm for Irinotecan Hydrochloride in water (a &b) and in serum/ plasma (c & d) at $\lambda_{excitation}$ of 374 nm.**

## 4.6 RESULTS AND DISCUSSION

**Estimation of Paclitaxel in liposomes**

Paclitaxel in methanol: chloroform (9:1) ratio yields a characteristic curve when scanned in the UV wavelength range between 200 to 400 nm. The scan shows absorption maxima at 229 and 273 nm. The absorptivity at 229 ± 2nm was found satisfactory and hence was selected as the analytical wavelength and used for further investigations. The regression equation was found to be y = 0.0318x + 0.0219. A correlation coefficient of 0.9993 (Table 4.1) indicated that absorbance and concentration of the drug were linearly related. Beer's law was found to be obeyed between 1-30 μg/ml (Table 4.1). The blank [methanol; chloroform (9:1)] does not interfere in the measurement of the paclitaxel absorbance.

The mean absorbance values of the methanol: chloroform solutions of Paclitaxel at different concentrations at preselected time intervals were determined. ANOVA studies of the results indicated no significant difference between the readings. Thus Paclitaxel is stable over a period of 24 h in methanol: chloroform mixture.

The presence of the other constituents of the liposomes such as DOPE, CHEMS, Cholesterol, mPEG-DSPE at the levels at these materials were included in the liposomes, did not interfere in the estimation of Paclitaxel.

The method was used to estimate Paclitaxel unentrapped and entrapped in the liposomes after the recovery of the liposomes by centrifugation. Good mass balance was obtained between the amount of Paclitaxel added and that recovered from the liposomes and unentrapped, signifying the suitability of the method for this application.

Paclitaxel in PBS pH 7.4 (with 20% methanol) and PBS 5.0 (with 20% methanol) gives a regression equation y = 0.0422x -0.0132 with correlation coefficient of 0.9996 and y = 0.0419x -0.0141 with correlation coefficient of 0.9993 respectively, was developed for estimating the release profile of Paclitaxel from conventional and pH sensitive 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.

High Performance Liquid Chromatography (HPLC) method was also developed for estimating Paclitaxel in the aqueous phase, using Acetonitrile: water (65:35 v/v) as mobile phase at wavelength 227nm. A standard curve was constructed for PCL by plotting calculated peak area or response factor versus standard stock concentrations. The adopted method demonstrated excellent linearity over the range 0.5 to 10 µg/ml in standard solutions. The calibration curve was observed to be linear in this range with correlation coefficients ($R^2$) consistently higher than 0.99 at the confidence limits of 90 to 110 %.

A regression equation of y = 0.6907x - 0.1399 and correlation coefficient of 0.9991 was obtained indicates the linearity. This method was further utilized for estimating the paclitaxel content in the stability study samples.

The estimation procedures are found to be reliable, accurate and suitable for formulation development.

**Estimation of Irinotecan Hydrochloride in liposomes**

Irinotecan Hydrochloride in methanol: chloroform (9:1) ratio yields a characteristic curve when scanned in the UV wavelength range between 200 to 400 nm. The scan shows absorption maxima at 226, 256, 361, 372 nm. The absorptivity at 361nm was found satisfactory and hence was selected as the analytical wavelength and used for further investigations. The regression equation was found to be $y = 0.0807 x + 0.0119$ with correlation coefficient of 0.9998 (Table 4.10) indicated that absorbance and concentration of the drug were linearly related. Beer's law was found to be obeyed between 1-10 μg/ml (Table 4.10). Experimental and calculated values for the method are presented in Table 4.14. The blank does not interfere in the measurement of absorbance of drug.

The mean absorbance values of the methanol: chloroform solutions of Irinotecan Hydrochloride at different concentrations at preselected time intervals were determined. ANOVA studies of the results indicated no significant difference between the readings. Thus Irinotecan Hydrochloride is stable over a period of 24 h in methanol: chloroform mixture. The presence of the other constituents of the liposomes such as DOPE, CHEMS, Cholesterol, mPEG-DSPE at the levels at these materials were included in the liposomes, did not interfere in the estimation of Irinotecan Hydrochloride.

The method was used to estimate the amount of Irinotecan Hydrochloride entrapped in liposomes obtained after the recovery of the liposomes by centrifugation.

Irinotecan in double distilled water gives a regression equation of $y = 0.0497 x + 0.0182$ with correlation coefficient of 0.9996 was developed for estimating the free or unencapsulated drug in the supernatant.

Good mass balance was obtained between the amount of Irinotecan Hydrochloride added and that recovered from the liposomes and unentrapped IH in supernatant, signifying the suitability of the method for this application.

Irinotecan in PBS pH 7.4 and PBS pH 5.0 gives a regression equation y = 0.0635 x - 0.0368 with correlation coefficient of 0.9994 and y = 0.0578 x - 0.0237 with correlation coefficient of 0.9996 respectively, was developed for estimating the release profile of Irinotecan from conventional and pH sensitive 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.

A Spectrofluorimetric method was also developed for the estimation of irinotecan in water and human plasma or serum with $\lambda_{excitation}$ = 374 nm and λemission = 435 nm. A. regression equation of y = 2.8293x + 4.2123 and correlation coefficient of 0.9996 in water and regression equation of y = 15.1x + 18.6 with correlation coefficient of 0.9995 in human plasma or serum. This method was further used for analyzing the serum stability of the pH sensitive liposomal formulations and also to assay the stability samples.

## 4.7 REFERENCES

Crosasso, P., Ceruti, M., Brusa, P., Arpicco, S., Dosio, F., Cattel, L. (2000) Preparation, characterization and properties of sterically stabilized paclitaxel-containing liposomes. *J. Contr. Rel.*, **63**: 19–30.

Elkharraz, K., Faisant, N. , Gusec, C., Siepmann, F., Arica-Yegin, B., Oger, J.M., Gust, R., Goepferich, A., Benoit, J.P., Siepmann, J. (2006) Paclitaxel-loaded microparticles and implants for the treatment of brain cancer: Preparation and physicochemical characterization. *Int. J. Pharm.* **314**: 127–136.

Kim, S. C., Yu, J., Lee, J. W., Park, E., Chi, S. (2005) Sensitive HPLC method for quantitation of paclitaxel (Genexol®) in biological samples with application to preclinical pharmacokinetics and biodistribution. *J. Pharm. Biomed. Anal.* **39** : 170–176.

Potineni, A., Lynn, D. M., Langer, R., Amiji, M. M. (2003) Poly (ethylene oxide)-modified poly (b-amino ester) nanoparticles as a pH-sensitive biodegradable system for paclitaxel delivery. *J Contr. Rel.* **86**:223-234.

Sadzuka, Y., Hirotsu, S., Hirota, S. (1999) Effective Irinotecan (CPT-11)-containing liposomes: Intraliposomal conversion to the active metabolite SN-38. Jpn. J. Cancer Res. **90**: 226-232.

Xie, Z., Guan, H., Chen, X., Lu, C., Chen, L., Hu, X., Shi, Q., Jing, X. (2007) A novel polymer–paclitaxel conjugate based on amphiphilic triblock copolymer. *J Contr. Rel.* **117**: 210–216.