Chapter 3
Analytical Method

3.1 Materials

Table 3.1 gives the list of materials and their sources.

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List of materials and their sources

Materials	Source
Etoposide and Cytarabine	Gift samples from Biocon, Bangalore
Poly (DL lactide-co-glycolide) PLGA	Gift sample from Boehringer Ingelheim
50:50 (inherent viscosity 0.22 dl/g)	Limited, Germany
Pluronic F- 68 (BASF)	Gift sample from Alembic Ltd, Vadodara
mPEG (MW 5000)	Sigma, USA
Chloroform, AR grade	SD Fine Chemicals, Mumbai
Methanol, AR grade	SD Fine Chemicals, Mumbai
Acetone, AR grade	SD Fine Chemicals, Mumbai
Dihydrogen phosphate	SD Fine Chemicals, Mumbai
Disodium hydrogen phosphate	SD Fine Chemicals, Mumbai
Sodium Chloride	SD Fine Chemicals, Mumbai

3.2 Estimation of Etoposide by UV Spectrophotometry

3.2.1 Calibration curve of Etoposide in Methanolic Phosphate Buffer Saline (3:7)

Methanolic PBS was prepared by addition of 30 ml AR grade methanol to 70 ml of PBS pH 7.4 in a 100 ml volumetric flask. Stock solution of 100 µg/ ml etoposide in methanolic PBS was prepared and was scanned to determine its $\lambda_{max.}$ From this stock solution, 1.0, 1.5, 2.0, 3.0 and 4.0 ml were accurately taken and transferred to 10 ml volumetric flask. The final volume was made up to 10 ml with methanolic PBS to prepare solutions containing 10, 15, 20, 30 and 40 µg/ml of etoposide in methanolic PBS. The solutions were mixed using vortex mixture and their absorbances measured at λ_{max} using methanolic PBS as blank on Shimadzu 1601 UV-Visible Spectrophotometer and calibration curve was plotted.

3.2.2 Calibration curve of Etoposide in Chloroform

Stock solution of 100 μg/ ml etoposide was prepared by dissolving 10 mg of drug in 100 ml of chloroform and was scanned in the range; 200-400nm to determine its λ_{max}. From this stock solution, different dilution having concentrations of 10, 20, 30, 40 and 50 μg per ml of etoposide in chloroform were prepared and a calibration curve was plotted using chloroform as blank on Shimadzu 1601 UV-Visible Spectrophotometer.

3.2.3 Estimation of Etoposide from PLGA, PLGA-mPEG or PLGA-PLURONIC Nanoparticles

100mg of drug loaded nanoparticles (PLGA NP, PLGA-mPEG NP or PLGA-PLURONIC NP) were taken and dissolved in 10 ml of chloroform. The dispersion was further subjected to centrifugation at 10,000 rpm for ten minutes for settling of any un-dissolved particles and the supernatant was analyzed for etoposide by UV-Visible Spectrophotometer. The analysis of drug in nanoparticles was carried out using the Blank NP dissolved in chloroform as blank in order to nullify the interference of the excipients.

3.2.4 Validation parameters

Validation was carried out by finding accuracy, precision and linearity (Connors et al., 1986) to determine the reproducibility and repeatability of the analytical method under the present operating conditions.

3.2.4.1 Accuracy and Precision

Accuracy of an analytical method is the closeness of test results obtained by that method to true value. Accuracy is calculated from the test results as the % analyte recovered by assay. For each concentration the % accuracy was calculated.

Precision of an analytical method is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple sampling of homogenous sample (Bolton, 1990). The precision of analytical method was expressed as the standard

deviation or relative standard deviation. The standard deviation was calculated from following formula:

$SD = [S(x - X)/ n - 1]½$

Where, x is an individual measurement in a set, X is arithmetic mean of the set and n is total number of replicated measurements taken in set.

3.2.4.2 Linearity

Linearity of an analytical method is its ability to elicit, test results that are directly or by a well defined mathematical transformation proportional to the concentration of analyte in samples with a given range (Hubert et al., 2003). Beer's law states that absorbance is proportional to the concentration of the absorbing species. A calibration curve was prepared by plotting the dependent variable (absorbance Y) as a function of the independent variable (Concentration X). This relation if found with a series of measurements.

$Y = mX + C$

Where m is a slope of line and C is the intercept on the Y axis.

Linearity was calculated from the above described methods. For each concentration the regression coefficient was calculated.

3.2.4.3 Stability and Selectivity

Stability of the solutions of etoposide used for preparing the calibration curves in the methods, was ascertained by observing for changes in the absorbances at their respective analytical wavelengths over a period of 72 hours.

Etoposide was estimated in the presence of other excipients of nanoparticles (PLGA, Pluronic F-68 and mPEG) in a 1:1 ratio to study the selectivity of the method.

3.2.5 Results and Discussion

Calibration curve of Etoposide in Methanolic Phosphate Buffer Saline (3:7)

ETO in methanolic PBS yields a characteristic spectrum in the ultraviolet range between 200 and 400 (Fig. 3.1) and it shows absorption maxima at 241 nm and 286 nm. The wavelength 286 nm avoids any possible interference in the lower UV range during estimation (Reddy and Murthy 2005). It was observed that absorption maxima at 241 nm shifted over a period of time where as the peak at 286 nm was stable and sharper. Hence, 286nm wavelength was chosen as the λ_{max}.

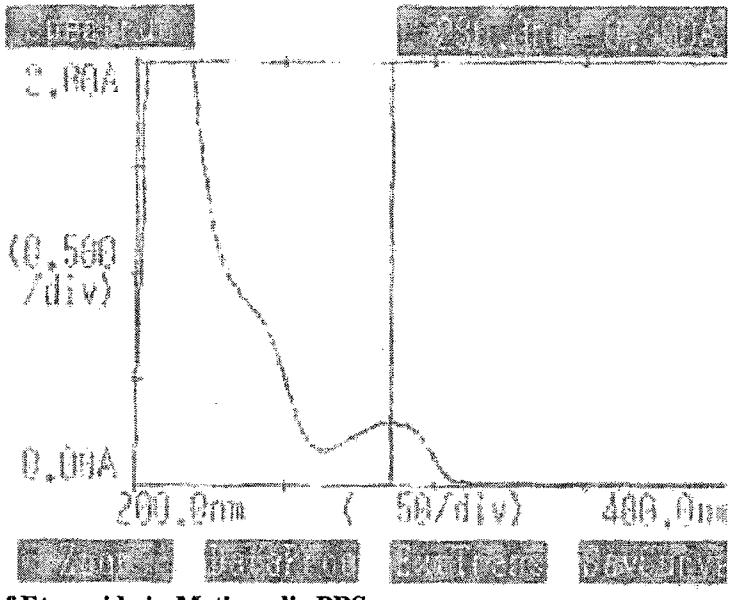

Fig. 3.1: Scan of Etoposide in Methanolic PBS

Table 3.2 shows the calibration data for estimation of etoposide in methanolic PBS (pH 7.4) along with the regressed values obtained by using the regression equation y = 0.0176x + 0.0098 having correlation coefficient (R^2) value of 0.9982. The calibration plot of etoposide in methanolic PBS is shown in Fig. 3.2, a high R^2 value of 0.9982 indicates linearity.

Table 3.3 show accuracy and precision for the method for estimation of etoposide in methanolic PBS by UV spectroscopy. The method shows percent accuracy in the range of 95 to 105%. The low RSD values indicate precision of the method. RSD below 2% is acceptable for research work

Table 3.2
Calibration data for estimation of Etoposide in Methanolic Phosphate buffer saline (pH 7.4)

Concentration (µg/ml)	Absorbance ± S.E.M	Regressed value*
10	0.190 ± 0.022	0.186
15	0.274 ± 0.014	0.274
20	0.362 ± 0.0074	0.362
30	0.523 ± 0.083	0.538
40	0.723 ± 0.016	0.714

* Using regression equation y = 0.0176x + 0.0098, Correlation Coefficient (R^2) =0.9982

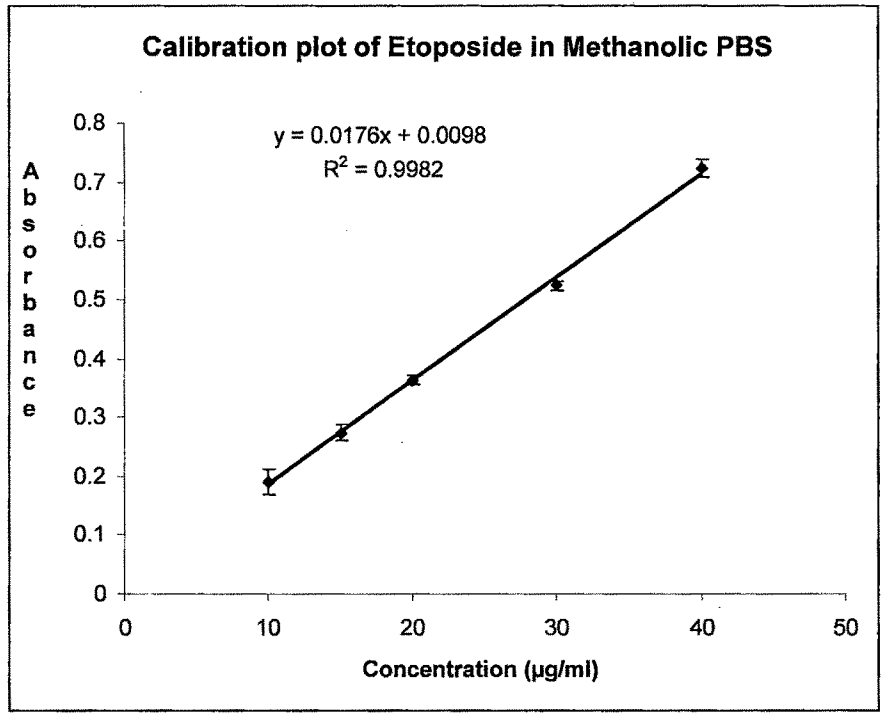

Fig. 3.2: Calibration Plot of Etoposide in Methanolic PBS

Table 3.3
Data for Accuracy and Precision for method of analysis of Etoposide in Methanolic PBS

Concentration added (µg/ml)	Concentration calculated (µg/ml)	Accuracy %	Precision (RSD)
10	10.41± 0.023	104.13± 0.418	0.220
15	14.45 ± 0.067	96.35 ± 0.336	0.463
20	20.01 ± 0.024	100.0 ± 0.488	0.119
30	30.31 ± 0.063	101.05 ± 0.259	0.207
40	39.62 ± 0.071	99.06 ± 0.700	0.179

RSD is Relative Standard Deviation = (standard deviation/mean concentration) x 100.

Table 3.4
Linearity data and regression analysis for Analytical method of estimation of Etoposide in Methanolic PBS

Data	Result
λmax	286 nm
Beer's law limit	10µg-40µg
Regression equation	y=0.0176x+ 0.0098
Regression coefficient (R^2)	0.9982

No significant difference between the amounts of drug added (actual) and observed concentration was noticed indicating accuracy of the method. Thus the method was found to be precise and linear in range of 5µg-40µg/ml. Table 3.4 shows details of the linearity data and regression analysis of the analytical method. Regression coefficient (R^2) was 0.9982, indicating a linear relationship between absorbance and concentration of the drug. Beer's law was obeyed between 10 to 40 µg ml per ml.

Table 3.5
Stability of ETO in Methanolic PBS over 72 hours and Selectivity in presence of Excipients.

Amount added (µg/ml)	Amount calculated over 72 h* (µg/ml) (Stability)	Accuracy %	Amount calculated with Excipients (µg/ml) (Selectivity)	Accuracy %
10	9.81± 0.013	98.100	9.93± 0.071	99.300
15	14.89 ± 0.042	99.267	14.93 ± 0.063	99.533
20	19.28 ± 0.011	96.400	19.81 ± 0.056	99.050
30	29.41 ± 0.038	98.033	29.92 ± 0.038	99.733
40	39.82 ± 0.062	99.550	40.02 ± 0.091	100.050

Stability was ascertained over a period of 72 hours. There was no significant difference between the mean absorbance values of the amount added and amount calculated after 72h (Table 3.5). The high % accuracy results in the range of 96 to 101% showed that the method was stable for estimation of ETO in methanolic PBS over 72h.

Selectivity study was carried out for checking the interference of the excipients with the drug. Out of the three main excipients in the formulation, two (PLGA and mPEG) were insoluble in methanolic PBS and hence were non interfering in the results. The third excipient Pluronic F-68 was added to drug in a 1:1 ratio. There was no significant difference between the mean absorbance values of the ETO with and without the excipient (Pluronic F-68). Hence it was concluded that the method was selective for estimation of ETO in methanolic PBS and there was no interference with the excipients used.

Calibration curve of Etoposide in chloroform

ETO in chloroform yields a characteristic spectrum in the ultraviolet range between 200 and 400 and it shows absorption maxima at 241 nm and 286 nm (Fig. 3.3). 286nm wavelength was selected as it was more stable, sharper and did not show interference in the lower UV range during estimation.

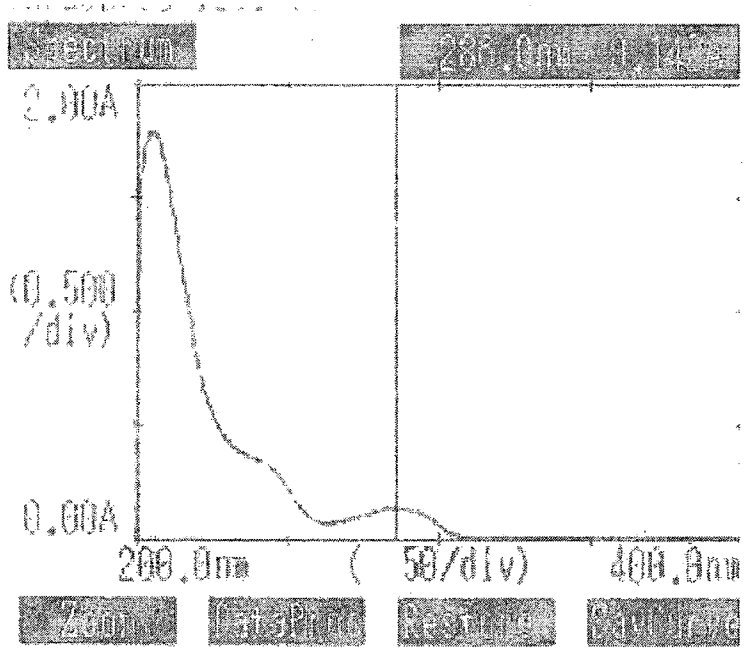

Fig. 3.3: Scan of Etoposide in chloroform

Table 3.6 shows the calibration data for estimation of etoposide in chloroform with regressed values obtained by using the equation y = 0.0081x -0.0002. Fig 3.4 shows the standard calibration plot of etoposide in chloroform. Table 3.7 show accuracy and precision values and it was seen that the method shows percent accuracy in the range of 95 to 102% with low RDS values indicating precision of the method. Table 3.8 gives linearity data and regression analysis for the analytical method for estimation of etoposide in chloroform. Regression coefficient (R^2) was 0.9998, indicating a linear relationship between absorbance and concentration of the drug. Beer's law was obeyed between 10 to 50 µg ml per ml.

Table 3.6
Calibration data for estimation of Etoposide in Chloroform

Concentration (µg/ml)	Absorbance ± S.E.M	Regressed value*
10	0.081 ± 0.013	0.081
20	0.162 ± 0.018	0.162
30	0.240 ± 0.024	0.243
40	0.320 ± 0.021	0.324
50	0.405 ± 0.011	0.405

* Using regression equation y = 0.0081x -0.0002, Correlation Coefficient (R^2) =0.9998

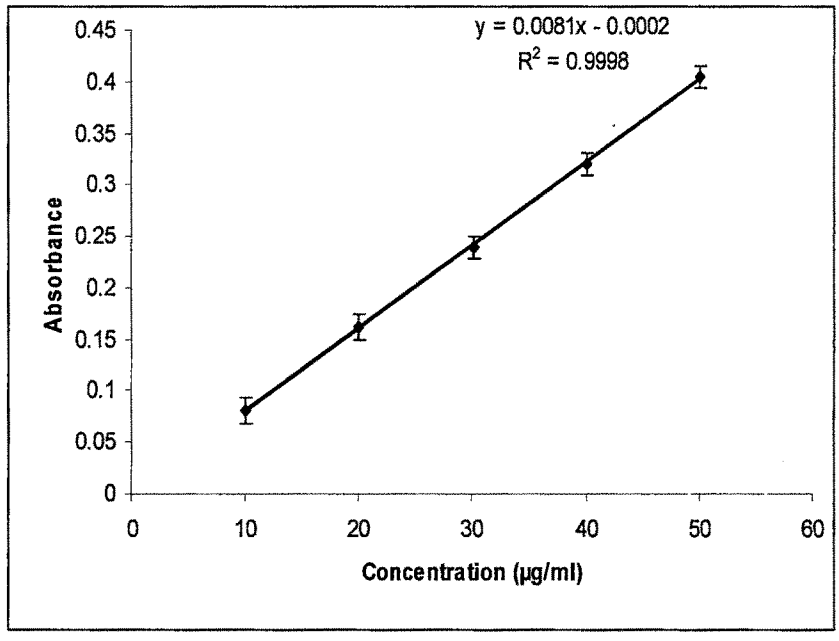

Fig. 3.4: Calibration Plot of Etoposide in Chloroform

Table 3.7
Data for Accuracy and Precision for method of analysis of Etoposide in Chloroform

Concentration added (µg/ml)	Concentration calculated (µg/ml)	Accuracy %	Precision (RSD)
10	10.21± 0.023	102.13± 0.41	0.225
20	20.01 ± 0.021	100.0 ± 0.40	0.104
30	30.31 ± 0.063	101.05 ± 0.25	0.207
40	39.21± 0.020	98.02± 0.12	0.051
50	50.30± 0.019	100.6± 0.05	0.037

RSD is Relative Standard Deviation = (standard deviation/mean concentration) x 100.

Table 3.8
Linearity data and regression analysis for Analytical method of estimation of Etoposide in Chloroform

Data	Result
λmax	286 nm
Beer's law limit	10µg-50µg
Regression equation	y=0.0081x-0.0002
Regression coefficient (R^2)	0.9998

Table 3.9
Stability of ETO in Chloroform over 72 hours and Selectivity in presence of Excipients.

Amount added (µg/ml)	Amount calculated over 72 h (µg/ml) (Stability)	Accuracy %	Amount calculated with Excipients (µg/ml) (Selectivity)	Accuracy %
10	10.11± 0.023	101.13± 0.41	9.89± 0.013	98.90± 0.21
20	20.00 ± 0.021	100.0 ± 0.10	19.89 ± 0.011	99.45 ± 0.20
30	30.21 ± 0.063	100.70 ± 0.25	30.10 ± 0.033	100.03 ± 0.15
40	39.19± 0.020	97.97± 0.12	38.90± 0.020	97.25± 0.22
50	50.20± 0.019	100.4± 0.05	50.10± 0.009	100.2± 0.02

Stability was ascertained over a period of 72 hours. There was no significant difference between the mean absorbance values of the amount added and amount calculated after 72h (Table 3.9). The results of high accuracy in the range of 97 to 102% showed that the method was stable for 72 h for estimation of ETO in chloroform.

For checking the interference of the excipients, selectivity study was carried out by addition of excipients (PLGA, Pluronic F-68 and mPEG) in a 1:1 mixture with ETO in chloroform. There was no significant difference between the mean absorbance values of the ETO added and amount of ETO calculated after addition of excipients. The results indicated that there was no interference of the excipients and the method was selective for estimation of ETO in chloroform.

3.3 Analytical method development for Cytarabine

There is no analytical method reported for cytarabine in Phosphate Buffer Saline (PBS) pH 7.4, hence the method was developed and validated.

3.3.1 Calibration curve of Cytarabine in Phosphate Buffer Saline (PBS) pH 7.4

10 mg of cytarabine was accurately weighed and transferred to 100 ml volumetric flask and PBS was added to make up the volume of the flask. From this stock solution (100 µg per ml of cytarabine in PBS) different dilutions having 5, 10, 20, 30, 40 and 50 µg/ ml of cytarabine in PBS were prepared. The solutions were mixed using vortex mixture and their absorbances measured at λ_{max} using PBS pH 7.4 as blank on Shimadzu 1601 UV-Visible Spectrophotometer and calibration curve was plotted.

3.3.2 Estimation of Cytarabine in PLGA and PLGA-MPEG NP

100mg of NPs were added to 10 ml of 1:1 mixture of chloroform and methanol. This dispersion was subjected to shaking at room temperature to ensure complete dissolution of the particles and the resulting solution was evaporated to dryness, and the dried residue was reconstituted with 5 ml of phosphate buffer saline. The reconstituted dispersion was centrifuged at 10000 rpm for 15 min. In this extraction procedure, the drug was solubilised in PBS and the polymer which was not soluble remained in the pellet. The supernatant was analyzed for drug using UV- spectroscopy at λ_{max} 271 nm using calibration curve of cytarabine in PBS as explained in section 3.3.1.

3.3.3 Validation parameters

Validation was carried out by finding accuracy, precision and linearity to determine the reproducibility and repeatability of the analytical method under the present operating conditions.

3.3.4 Results and Discussion

CYT in PBS (pH 7.4) yields a characteristic spectrum when scanned in the ultraviolet range between 200 and 400 nm. The scan (Fig. 3.5) shows absorption maximum at 271 nm and this wavelength was chosen as the analytical wavelength. Correlation coefficient (R^2) for developed method was found to be 0.9991 signifying that a linear relationship existed between absorbance and concentration of the drug. Beer's law was obeyed between 5 and 50µg/ml. Regression analysis was performed on the experimental data. The raw data along with the results of the regression analysis is shown in Table 3.11, respectively. Regression equation for standard curve was y = 0.0184x - 0.003 (Fig.3.6). Parameters for the spectrometric method of analysis for CYT are shown in Table 3.12.

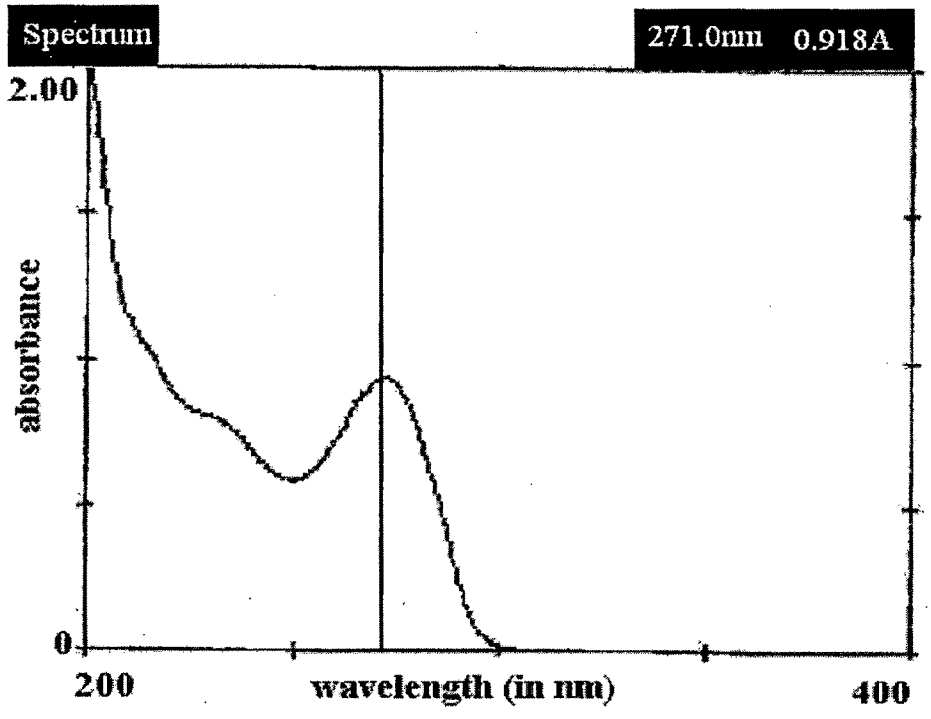

Fig. 3.5: Scan of Cytarabine in PBS

Table 3.10
Calibration curve for estimation of cytarabine in PBS (pH 7.4)

Concentration (µg/ml)	Absorbance ± S.E.M	Regressed value*
5	0.092 ± 0.0032	0.089
10	0.192 ± 0.0059	0.181
20	0.359 ± 0.0092	0.365
30	0.571 ± 0.0048	0.541
40	0.742 ± 0.0052	0.733
50	0.918 ± 0.0062	0.917

* Using regression equation y = 0.0184x - 0.003, Correlation Coefficient (R^2) =0.9991

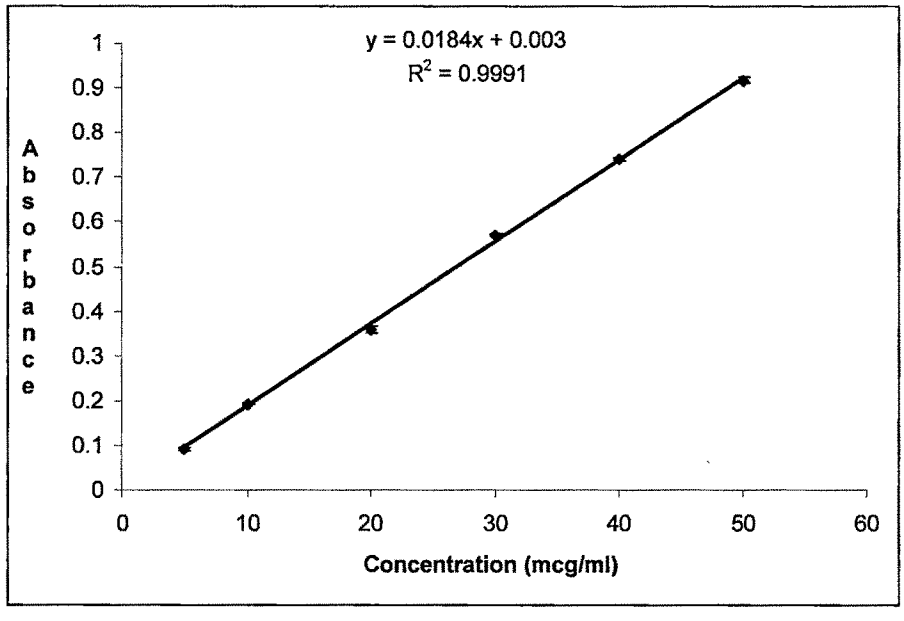

Fig. 3.6: Calibration curve of Cytarabine in PBS (pH 7.4)

Table 3.11
Parameters for UV spectrometric method of analysis of CYT in PBS

Parameters	Results
λ_{max}	271 nm
Linearity range	5-50 µg/ml
Regression equation	y = 0.0184x - 0.003
Correlation coefficient (R^2)	0.9991

Table 3.12 show accuracy and precision for the CYT estimation by UV spectroscopy. Low RSD values below 1% indicated precision of the method. No significant difference between the amount of drug added (actual) and observed concentration was noticed indicating accuracy of the method (Boulangeret al., 2003; Guidance for industry, 2001.).

Table 3.12
Accuracy and Precision for the Cytarabine estimation using PBS by UV spectroscopy.

Standard concentration (µg/ml)		Accuracy (%)	Precision (RSD)*
Actual	Observed		
5	4.98 ±0.012	99.6	0.240
10	10.12 ±0.083	101.2	0.820
20	19.98 ±0.095	99.9	0.475
30	30.06±0.029	100.2	0.096
40	39.19±0.074	97.9	0.188
50	50.08 ±0.092	100.3	0.183

*RSD is Relative Standard Deviation = (standard deviation/mean concentration) x 100.

Table 3.13
Stability of CYT in PBS over 72h and Selectivity in presence of Excipients.

Amount added (µg/ml)	Amount calculated over 72 h (µg/ml) (Stability)	Accuracy %	Amount calculated with Excipients (µg/ml) (Selectivity)	Accuracy %
10	10.11± 0.023	101.13± 0.41	9.89± 0.013	98.90± 0.21
20	20.00 ± 0.021	100.0 ± 0.10	19.89 ± 0.011	99.45 ± 0.20
30	30.21 ± 0.063	100.70 ± 0.25	30.10 ± 0.033	100.03 ± 0.15
40	39.19± 0.020	97.97± 0.12	38.90± 0.020	97.25± 0.22
50	50.20± 0.019	100.4± 0.05	50.10± 0.009	100.2± 0.02

Table 3.13 shows stability of CYT in PBS over 72 hours. There was no significant difference between the mean absorbance values of the amount added and amount calculated after 72h. The % accuracy values were high in the range of 97 to 102%. The results indicated that the method was stable for estimation of CYT in PBS for over 72h.

For checking the interference of the excipients with the drug, method for selectivity for estimation of CYT in PBS was carried out. There were three main excipients in the formulation, out of which two (PLGA and mPEG) were insoluble in PBS and hence were non interfering in the results. The third excipient (Pluronic F-68) was added in a 1:1 ratio in PBS with CYT. There was no significant difference between the mean absorbance values of the CYT with and without the excipient (Pluronic F-68). Hence it was concluded that the method was selective for estimation of cytarabine in PBS and there was no interference with the excipients used.

REFERENCES

Bolton S, Swarbrick J (1990). Basic definitions and concepts In: *Pharmaceutical statistics: Practical and clinical applications*, 3rd ed., Marcel Dekker, New York, pp 24.

Boulanger B, Dewe W, Chiap P, Crommen J, Hubert PH (2003). An analysis of the SFSTP guide on validation of bioanalytical methods: progress and limitations. *Journal of Pharmaceutical and Biomedical Analysis 32,* 753-765.

Connors KA, Amidon GL, Stella VJ (1986). *Chemical Stability of Pharmaceuticals: A Handbook for Pharmacists*. John Wiley and Sons Inc., New York, pp 561-576.

Guidance for industry (2001). Bioanalytical Method Validation, U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER).

Hubert PH, Chiap P, Crommen J, Boulanger B, Chapuzet E, Mercier N, Bervoas MS, Chevalier P, Grandjean D, Lagorce P, Lallier M, Laparra MC, Laurentie M, Nivet JC (2003). Validation of the quantitative analytical procedures.Harmonization of the steps. *S.T.P. Pharma practice 13*, 101-138.

Reddy H and Murthy RSR. (2005). Etoposide-Loaded Nanoparticles Made from Glyceride Lipids: Formulation, Characterization, in Vitro Drug Release, and Stability Evaluation. *AAPS PharmSciTech 6 (2)*, Article 24.