CHAPTER 3: ANALYTICAL METHOD DEVELOPMENT

The analytical methods employed during preparation and optimization of pDNA complexed lipoplexes and or estimating the marker β galactosidase transgene expression are discussed below. Further, analytical methods for estimating Etoposide and Docetaxel during their liposomal and lipoplex preparation, optimization and characterization are also discussed.

3.1 Estimation of plasmid DNA (pDNA)

Spectrophotometric analysis serves to be quickest, promising and reliable method or routine analytical needs. The estimation of pDNA by UV visible spectrophotometry in water was based on the observation that, plasmid DNA absorbs strongly in water and aqueous buffer as Tris buffer with maxima at 260 nm. The plasmid was quantified spectrophometrically in Tris buffer pH 8.0 at 260 nm. The calibration curve of the plasmid was constructed as shown in **figure 3.1.** The initial concentration of isolated plasmid was determined by the estimation at 260 nm i.e.

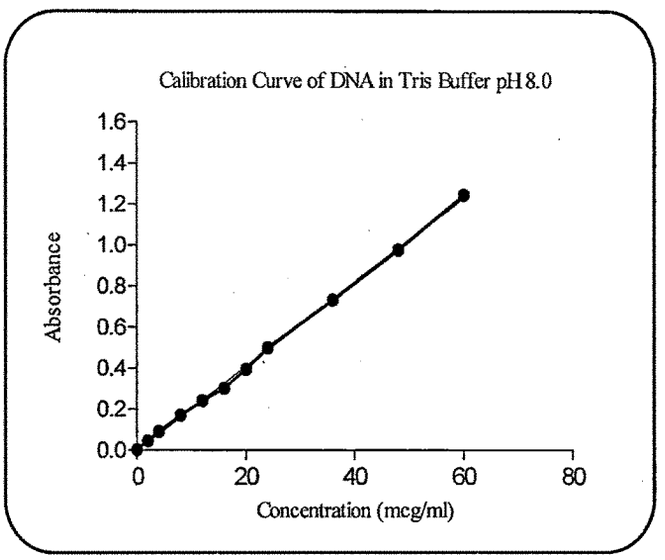
Absorbance of 1 is equivalent to concentration of 50 µg /ml

Briefly, suitable aliquots of initial concentrate was with drawl into 5 ml measuring cylinder and the volume was made up with Tris buffer pH 8.0 to give final concentrations of 2-60 µg/ml. The solutions were shaken well and the absorbance was measured at 260 nm using Tris buffer as blank by UV Visible Spectrophotometer (Schimadzu 1601). The results are as shown in **figure 3.1 below.**

Although the preparations of DNA absorb at 260 nm, the RNA also absorb more strongly in the same region. Proteins absorb maximally at 280 nm but also significantly at 260 nm. The other contaminants as phenol, chloroform may also interfere with DNA preparations and estimation. Therefore an accurate determination for purity of double stranded plasmid DNA devoid of RNA and proteins is required. The ratio of 260 to 280 nm provides an estimate of the plasmid purity. For pure DNA preparation, the ratio is in between 1.8 to 2.0. Higher and lower ratio indicates contamination with RNA and proteins respectively. [C.R. Middaugh (1998)].

The DNA solution in Tris buffer shows a absorption maximum at 260 nm and the correlation coefficient of 0.9991, which indicates that the absorbance and concentration of DNA are linearly related. The slope of the regressed line 0.0205 indicates moderate sensitivity of the method. The method demonstrated linearity of the curve up to the range 2-60 µg / ml concentration.

Concentration in mcg / ml	Absorbance ± SEM (n=6)
0	0.003 ± 0.0020
2	0.046 ±0.0010
4	0.092 ±0.0020
8	0.171 ±0.0020
12	0.242 ±0.0010
16	0.303 ±0.0010
20	0.396 ±0.0020
24	0.500 ±0.0040
36	0.731 ±0.0050
48	0.976 ±0.0050
60	1.245 ±0.0040



Absorbance Max: 260 nm

Beer's Law limit: 2-60 µg/ml

Regression Equation: y = 0.0205x - 0.0011

Regression Coefficient: $R^2 = 0.9991$

Figure 3.1: Calibration curve of plasmid DNA in Tris buffer pH 8.0

3.2 Estimation of β-Galactosidase:

β-Galactosidase protein estimation method presents the classical colorimetric assay for estimation of β-Galactosidase gene expression [J. Miller (1972)]. It provides a simple, reliable and economical quantitative estimate of β-gal expression by measuring enzyme activity directly. The expressed β-Galactosidase protein is able to hydrolyze the galactose sugar bond. In the assay described, the artificial chromogenic substrate ortho-nitrophenyl-β-D-galactopyranoside (ONPG) is used, which is cleaved at β-1-4-Glycosidic bond between 2-nitrophenol and galactose leading to release of orthonitrophenol **(Figure 3.2)**. ONPG is colorless, while the hydrolyzed product ortho-nitrophenol has a yellow color and absorbs light at 405 nm. The intensity of the color developed directly correlates with the quantity of β galactosidase expressed in the cell.

β-Galactosidase estimation was performed in 96 well plate. Fresh stock solution of β-Galactosidase (140 Unit/mg Stock - Sigma Aldrich) was prepared by diluting 10 µl of 1 Unit/ µl (7.14 µg/ µl) to 990 µl of lysis buffer (0.25 M Tris-HCl, pH 8.0, 0.5 % Nonidet –P40) and

vortexed to make 1:1000 stock solution. Using this stock solution, 50 µl of each β-Galactosidase standards per well were prepared as below:

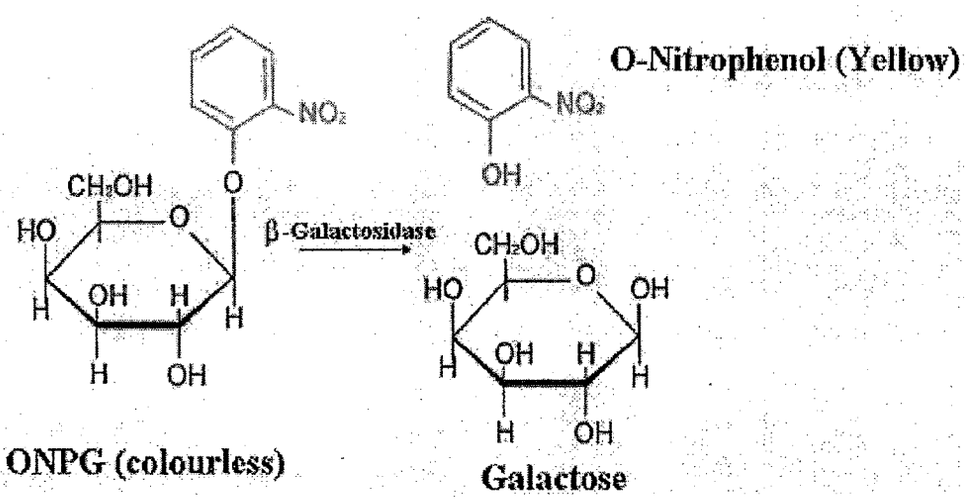


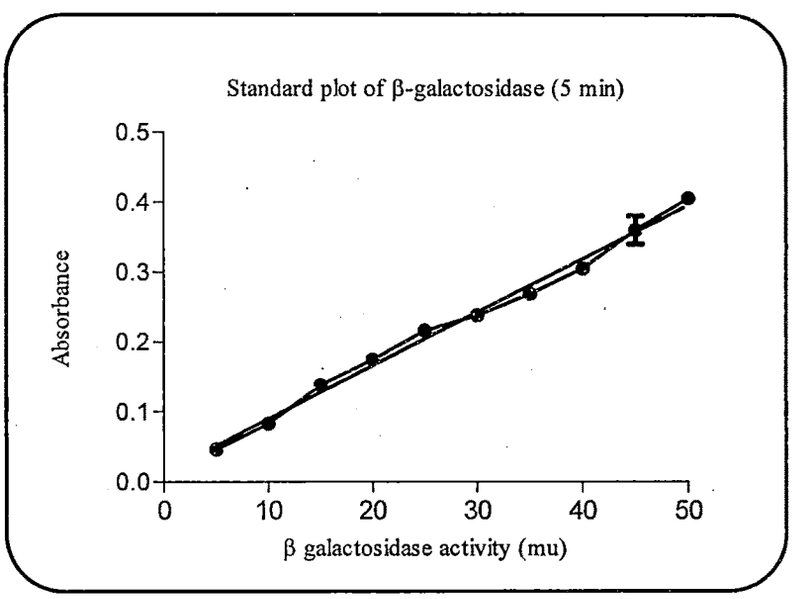
Figure 3.2: Hydrolysis of ONPG (colorless) to O-Nitrophenol (Yellow color) and galactose.

Table 3.1: Preparation of working standard solution of β galactosidase from stock solution

β galactosidase working standard (miliunits)	Volume of 1:1000 stock solution	Volume of lysis buffer
0	0	50
5	5	45
10	10	40
15	15	35
20	20	30
25	25	25
30	30	20
35	35	15
40	40	10
45	45	5
50	50	0

To this 50 µl of standard solution in lysis buffer, 50 µl of substrate solution (1.33 mg /ml ONPG (Sigma-Aldrich), 0.2 M sodium phosphate, pH 7.3 and 2 mM magnesium chloride was added in well plate. All samples were mixed by pipetting the well contents and were incubated at 37^0C. Absorbance was read after 5 min and 20 min at 405 nm using ELISA plate reader. The experiments were performed in triplicate and the values were reported as mean ± SD.

mU of Enzyme	Absorbance ± SEM (n=3)
5	0.046 ± 0.002
10	0.083 ± 0.003
15	0.138 ± 0.006
20	0.175 ±0.003
25	0.216 ± 0.004
30	0.238 ± 0.006
35	0.269 ± 0.006
40	0.305 ± 0.003
45	0.360 ± 0.02
50	0.405 ± 0.005

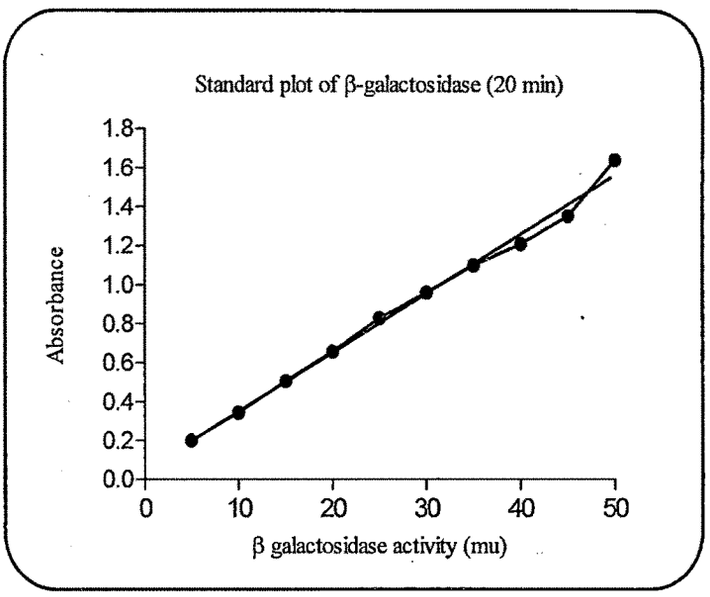


Absorbance Max: 405 nm
Beer's Law limit:: 5-50 mu
Regression Equation: y = 0.0078 x + 0.013
Regression Coefficient: $R^2 = 0.993$

Figure 3.3: Calibration curve of β galactosidase by reacting known quantity of β gal (mu) with ONPG after 5 minutes

The readings showed linear increment in absorbance with time. The correlation coefficient of 0.993 after 5 and 20 minutes of absorbance reading indicated that, the absorbance and concentration of ONP were linearly related. The absorbance of ONP is converted to β galactosidase activity and is used for the estimation of transfection efficiency of various transecting agents in vitro on cell lines after cell lysis with lysis buffer.

mU of Enzyme	Absorbance ± SEM (n=3)
5	0.200 ± 0.004
10	0.343 ± 0.003
15	0.505 ± 0.002
20	0.657 ± 0.005
25	0.829 ± 0.004
30	0.960 ± 0.003
35	1.100 ± 0.004
40	1.210 ± 0.003
45	1.352 ± 0.05
50	1.640 ± 0.006



Absorbance Max: 405 nm

Beer's Law limit: 5-50 mu

Regression Equation: $y = 0.0303 x + 0.046$

Regression Coefficient: $R^2 = 0.993$

Figure 3.4: Calibration curve of β galactosidase by reacting known quantity of β gal (mu) with ONPG after 20 minutes

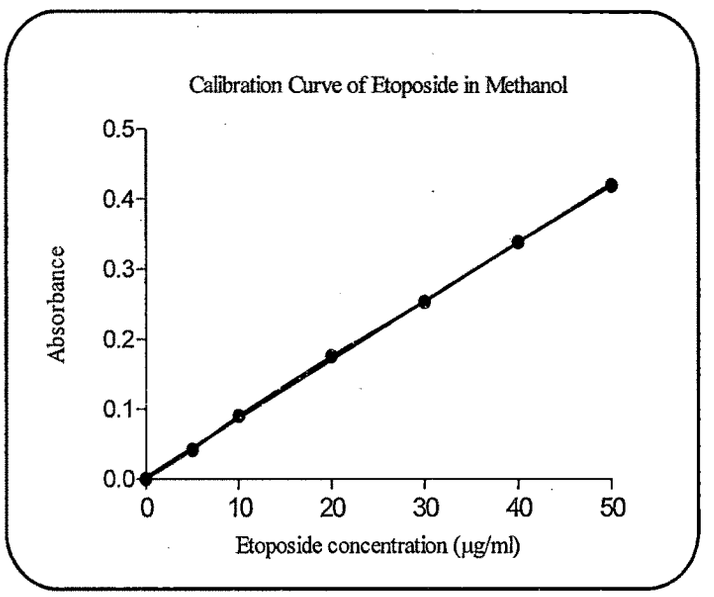
3.3 Estimation of Etoposide:

The spectroscopic determination of Etoposide (ETP) in the liposomes for routine analysis is based absorption spectrum of ETP in UV-Visible region after dissolving in Methanol. Etoposide was observed to show absorption maximum at 284 nm in methanol. A common method or estimation of ETP during entrapment efficiency and estimation of *in vitro* drug release from liposomes determination was developed in methanol.

Stock solution of 100µg/ml of ETP was prepared by dissolving 10 mg of Etoposide in 100 ml methanol. The aliquots of standard stock solution were diluted serially with sufficient methanol to obtain concentration range of 5 to 50 µg/ml. A calibration curve for ETP was obtained by measuring the absorbance at 284 nm against methanol as a blank. The experiment was repeated on three consecutive days using freshly prepared stock solutions each time. Mean values (n=3)

along with the standard error mean are recorded below. The regressed values of absorption were plotted graphically against the concentration, as shown in Figure 3.5. Stability of the solutions of Etoposide in Methanol used for preparing the calibration curve was ascertained by observing the changes in the absorbance of the solution at the analytical wavelength, over a period of 48hr at freeze temperature.

Etoposide Concentration (µg/ml)	Absorbance ± SEM (n=3)
0	0
5	0.042 ± 0.0011
10	0.090 ± 0.0021
20	0.176 ± 0.0011
30	0.253 ± 0.0005
40	0.339 ± 0.0011
50	0.420 ± 0.0015



Absorbance Max: 284 nm
Beer's Law limit: 0 – 50 µg/ml
Regression Equation: y=0.0084 x + 0.0029
Regression Coefficient: R^2 = 0.9996

Figure 3.5 Calibration Curve of Etoposide in Methanol at 284 nm.

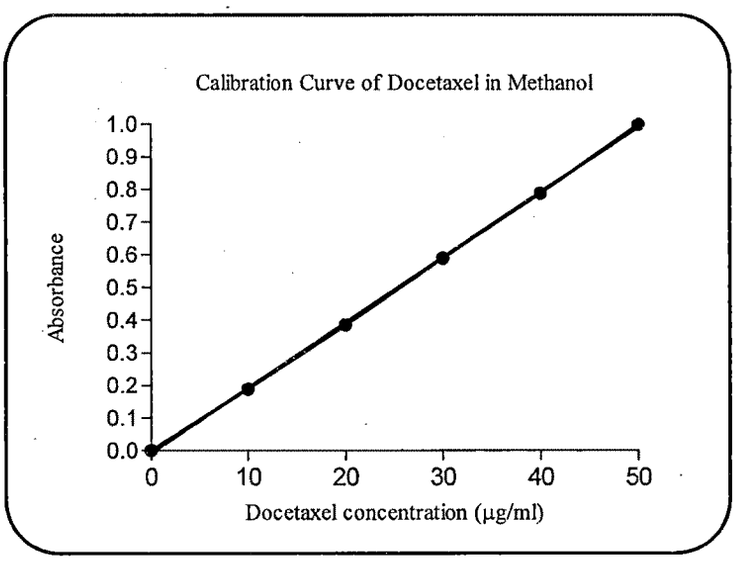
ETP showed linear increment in absorbance with concentration in the range of 0 to 50 µg/ml. The regression coefficient of 0.9996 confirmed the linearity. The measurements in successive days showed reproducible results indicated by low SEM values demonstrating accuracy and precision of the method. Further, the absorbance of ETP solution at all concentration in methanol at 4^0 C was unchanged for 48 hrs suggesting stability of drug in the estimation medium.

3.4 Estimation of Docetaxel Trihydrate:

The spectroscopic determination of Docetaxel Trihydrate (DTX) in the liposomes for routine analysis has based on absorption spectrum of DTX in UV-Visible region after dissolving in Methanol. DTX was observed to show absorption maximum at 229 nm in methanol. A common method for estimation of DTX during entrapment efficiency and estimation of in vitro drug release from liposomes determination was developed in methanol.

Stock solution of 100µg/ml of DTX was prepared by dissolving 10 mg of DTX in 100 ml methanol. The aliquots of standard stock solution were diluted serially with sufficient methanol to obtain concentration range of 10 to 50 µg/ml. A calibration curve for DTX was obtained by measuring the absorbance at 229 nm against methanol as a blank. The experiment was repeated on three consecutive days using freshly prepared stock solutions each time. Mean values (n=3) along with the standard error mean are recorded below. The regressed values of absorption were plotted graphically against the concentration, as shown in **figure 3.6**. Stability of the solutions of DTX in Methanol used for preparing the calibration curve was ascertained by observing the changes in the absorbance of the solution at the analytical wavelength, over a period of 48hr at freeze temperature.

Etoposide Concentration (µg/ml)	Absorbance ± SEM (n=3)
0	0
10	0.189 ± 0.0012
20	0.385 ± 0.0023
30	0.589 ± 0.0014
40	0.788 ± 0.0024
50	0.999 ± 0.0018



Absorbance Max: 229 nm
Beer's Law limit: 0 – 50 µg/ml
Regression Equation: y=0.020 x - 0.016
Regression Coefficient: $R^2 = 0.9993$

Figure 3.6 Calibration Curve of Docetaxel in Methanol at 229 nm.

DTX showed linear increment in absorbance with concentration in the range of 0 to 50 µg/ml. The regression coefficient of 0.9993 confirmed the linearity. The measurements in successive days showed reproducible results indicated by low SEM values demonstrating accuracy and precision of the method. Further, the absorbance of DTX solution at all concentration in methanol at 4^0 C was unchanged for 48 hrs suggesting stability of drug in the estimation medium.

3.5 References:

1. C,R, Middaugh, R.K. Evans, D.L. Montgomery, D.R. Casimiro (1998) Analysis of plasmid DNA from a pharmaceutical perspective. J. Pharm. Sci. 87:130-146

2. J. Miller (1972). Experiments in molecular genetics. Cold Spring Harbor Laboratory: Cold Spring Harbor, New York. PP 352-355.