Chapter 3

Analytical Methods

Chapter 3

3.1 Introduction

Analytical methods required in successful development of lipoplex formulations were developed and partially validated for suitability of analysis. Analytical methods developed are divided in the following

1. Analytical methods used for quantification of pDNA
2. Analytical methods used for characterization of lipids

3.2 Analytical methods used for quantification of pDNA

Three methods were used for quantification of pDNA namely, UV spectrophotometric method, Spectrofluorometric method and Gel electrophoresis. Gel electrophoresis method will give instant idea on the complexation efficiency as well as help in identification of the isolated pDNA. Spectrofluorometric method and UV spectrophotometric method will give accurate determinations of the complexation efficiencies as well as help in other studies like serum stability studies.

3.2.1 UV Spectrophotometric Analysis of DNA

Estimation of the pDNA can be carried out by estimation of the ultraviolet absorbance. For DNA, the three main wavelengths of interest are 260 nm, 280 nm and 230 nm. Absorbance at 260 nm gives estimate of the amount of DNA present in the sample. Concentration can be calculated using the reading at 260 nm wavelength and a conversion factor based on the extinction coefficient for nucleic acid. Simultaneously, absorbance measurements at that wavelength on 280 nm can be used to estimate the amount of protein contaminant in the sample based on presence of aromatic amino acids which absorb light at 280 nm. Measurement at 230 nm can used to determine the amount of siRNA that may be present in the samples. In addition, an absorbance reading at 320 nm takes into account any light-scattering components in the sample. The reading at 320 nm wavelength is subtracted from the 260 nm, 280 nm and 230 nm values as background. Results of known dilution of DNA solution gave the good linearity and reproducibility. This range of linearity will be used in the further studies to find out the concentration of pDNA.

3.2.1.1 Material

1. **DNAse free water (DFW):**

   DFW was prepared by autoclaving double distilled water at 121°C and 15 Psi for 15 min. For preparation of stock and subsequent dilution of the pDNA for spectroscopic estimation, DNAse free water (DFW) was used.

3.2.1.2 Method

pDNA was quantified using a UV spectrophotometric method (1, 2). Absorbance of the solution of the pDNA was checked by taking the absorbance values at four wavelengths i.e. 230 nm, 260 nm, 280 nm using NanoDrop 2000 instrument (NanoDrop, Germany). Absorbance values at all the wavelengths were corrected for scattering from sample by substracting the absorbance value at 320 nm (correction performed automatically by software). Purity of the pDNA was determined by evaluating the ratio of A_{260}/A_{280} and A_{260}/A_{230}. Once DNA was confirmed for its purity, calibration curve was constructed. pDNA stock solution of 1 µg/µL was prepared in DFW and by appropriate dilutions pDNA solutions of various concentrations between 2 ng/µL to 200 ng/µL were prepared. Absorbance values of these solutions were recorded at 260 nm NanoDrop UV spectrophotometer. Content of pDNA was calculated by corrected absorbance at 260 nm i.e. A_{260}-A_{320} and multiplying the reading by dilution factor and using the relationship that A_{260} of 1.0 = 50 µg of dsDNA. Whole experiment was performed in triplicate. Graph of observed concentration versus actual concentration of DNA was plotted to find out linearity of specific concentration range and reproducibility of results.

3.2.1.3 Results and discussion

a) Purity of the pDNA

Absorption profile of the isolated pDNA is shown in **figure 3.1.** To estimate purity of DNA, the ratio of the A_{260} with A_{280} and A_{230} were considered as key parameters. Typical A_{260}/A_{280} ratio or pure DNA is between 1.8–2.0, while A_{260}/A_{230} ratio is generally 2.0-2.2. A_{260}/A_{280} and A_{260}/A_{230} values were 1.83 and 2.17 respectively suggested high purity of DNA **(table 3.1)**.

Table 3.1 Absorbance profile of pDNA

Wavelength (nm)	Mean optical density (Absorbance)	
230	0.137	Dilution factor (DF)= 250
260	0.299	
280	0.151	Conc. (µg/ml) = A260x 50µg/ml x DF
320	-0.002	
A_{260}/A_{280}	1.98	**Conc. mg/ml or µg/µl of isolated plasmid DNA = 3.74**
A_{260}/A_{230}	2.18	

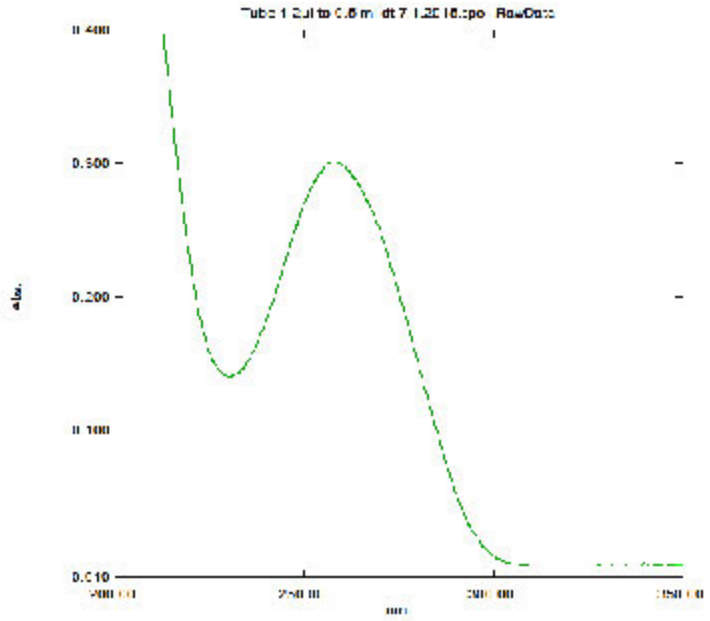

Figure 3.1 UV absorption profile of isolated pDNA

b) Calibration curve of the pDNA

Pure pDNA was then used to verify the correlation of actual concentrations and observed concentration Observed responses showed linear relationship with R^2 value of 1 between the range of 2 ng/µL to 200 ng/µL (**figure 3.2**). This was further used to record the range of linearity which will be useful in determination of DNA concentration in further studies.

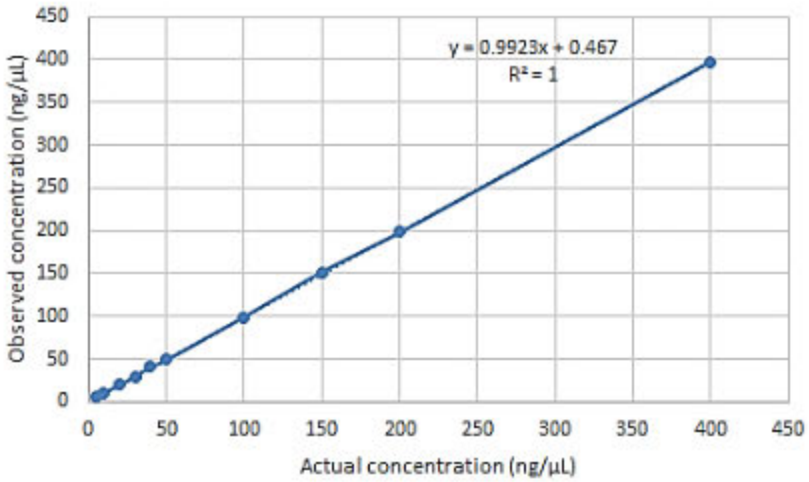

Figure 3.2 Correlation of actual concentration of pDNA vs observed concentration

c) Accuracy and Precision of the method

Accuracy and precision in terms of percentage recovery and percentage relative standard deviation (%RSD) respectively was evaluated for NanoDrop UV-spectrophotometric estimation method for the pDNA. Solution of pDNA in the concentration range of 5, 10, 20, 25, 50, 100, 150 and 200 ng/µL were prepared that covered the expected concentration range in the sample. Absorbance of each solution was recorded and % recovery was calculated as per following formula.

% DNA recovered = (Content of DNA after being recovered / theoretical content of the pDNA in sampled solution) x 100%.

The determination of the reproducibility of the method was determined from the absorbance of each sample at different time points and calculating the RSD. **Table 3.2 and table 3.3** represent accuracy, intraday and interday precision of the method, respectively. As it can be seen, the % recovery was found to be between 98.8% to 100.0% and the % RSD values were less than 2% conforming to the requirements of ICH guidelines (3, 4).

Table 3.2 Accuracy of the UV spectrophotometric method

Actual Concentration (ng/µL)	Observed Concentration (ng/µL)	Standard Deviation (SD)	%Recovery
10	09.86	0.023	98.60
100	99.32	0.156	99.32

200	198.9	0.282	99.45

*Values are represented as mean±SD, n=3.

Table 3.3 Interday and intraday precision of the UV spectrophotometric method

Actual Concentration (ng/µL)	Observed Concentration ± SD		%Relative Standard Deviation	
	Intraday precision	Interday Precision	Intraday precision	Interday Precision
10	9.92±0.021	9.87±0.02	0.21	0.20
100	99.32±0.29	98.96±0.11	0.29	0.11
200	198.90±0.31	199.25±0.26	0.15	0.13

*Values are represented as mean ± SD, n=3.

3.2.2 Spectrofluorometric method: Quantifluor

The QuantiFluor® dsDNA System(a) contains a fluorescent DNA-binding dye (504nmEx/531nmEm) that enables sensitive quantitation of small amounts of double-stranded DNA (dsDNA) in a purified sample. The assay is highly selective for dsDNA over other nucleic acids and is linear over a range of 0.05–200ng of dsDNA input (0.05–200ng/µl from 1µl of original sample). Lower dsDNA concentrations may be quantitated adjusting the standard curve accordingly.

3.2.2.1 Protocol for Quantitating dsDNA The manufacturers protocol was followed for performing calibration curve of the dsDNA.

3.2.2.2 Results and Discussion

a) Calibration curve of the pDNA

Pure pDNA was then used to verify the correlation of actual concentrations and observed concentration Observed responses showed linear relationship with R^2 value of ~1 between the range of 0.05 ng/µL to 200 ng/µL (**Figure 3.3 and Table 3.4**). This was further used to record the range of linearity which will be useful in determination of DNA concentration in further studies.

Table 3.4: Representative Data for the dsDNA Standard Curve and QuantiFluor® dye.

pDNA amount (ng/well)	Average fluorescence (RFU)
0	0
0.05	40±2
0.2	161±4
0.78	645±5
3.1	2570±68
12.5	10244±379
50	42974±896
100	82689±2101
200	160116±3014

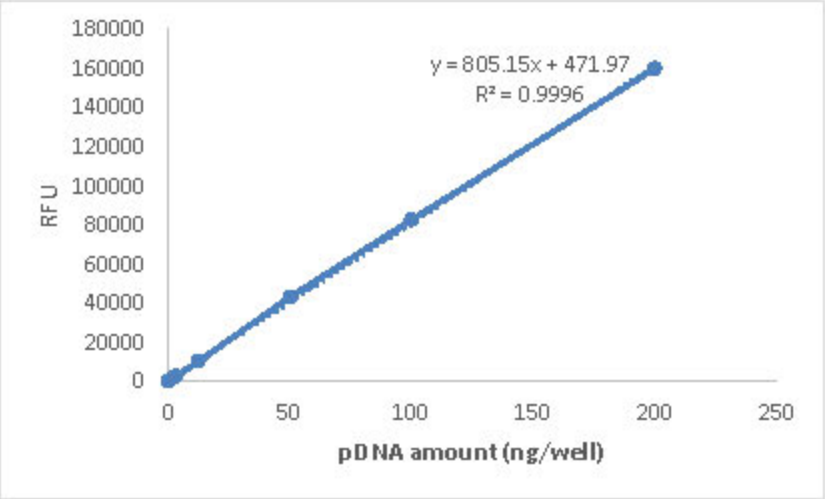

Figure 3.3 pDNA standard curve using spectrofluorimetry

c) Accuracy and Precision of the method

Accuracy and precision in terms of percentage recovery and percentage relative standard deviation (%RSD) respectively was evaluated for the pDNA. Solution of pDNA in the concentration range as done for calibration curve were prepared that covered the expected concentration range in the sample. Absorbance of each solution was recorded and % recovery was calculated as per following formula.

% DNA recovered = (Content of DNA after being recovered / theoretical content of the pDNA in sampled solution) x 100%.

The determination of the reproducibility of the method was determined from the absorbance of each sample at different time points and calculating the RSD. **Table 3.5** represent accuracy of the method. As it can be seen, the % recovery was found to be between 99.0% to 99.55% and the % RSD values around 2% conforming to the requirements of ICH guidelines.

Table 3.5 Accuracy of the Spectrofluorimetric method

Actual Concentration (ng/µL)	Observed Concentration (ng/µL)	Standard Deviation (SD)	%Recovery
0.05	0.0495	0.0020	99.00
50	49.52	1.63	99.04
200	199.1	2.1	99.55

*Values are represented as mean±SD, n=3.

3.2.3 Gel Electrophoresis of pDNA

The migration of free DNA under the influence of electric potential occurs in agarose gel electrophoresis which was utilized for its relative quantitation (5). This technique makes use of a DNA binding dye ethidium bromide (EtBr) that intercalates at the minor groove of the double strand of the nucleic acid which enables the visualization of the nucleic acid on the gel and complexation efficiency of the DNA with the cationic polymer. The basic principle is the separation of the nucleic acid based on their size. Smaller molecules migrate faster compared to the larger molecules from cathode towards anode in the electrophoretic chamber as the potential difference is applied. Lipids modified with Boc protected amino acids (Boc-amino acids) and cationic liposomes were prepared to complex with DNA effectively to make them useful for successful delivery of DNA. The complexation between the phospholipid and the DNA will lead to a retardation in the migration speed of the complex which gives a direct idea of the amount complexed compared to the quantity of the free DNA.

3.2.2.1 Material

1. **TAE buffer (Tris/Acetate/EDTA buffer, pH 8.4)**

   50X TAE buffer:

   24.2 g Tris free base and 1.861 g Disodium EDTA were dissolved in 70 mL of double distilled water. 5.71 mL acetic acid was added and solution was adjusted to 100 mL volume using double distilled water. Buffer was stored at 2-8 °C in refrigerator.

   1X TAE buffer:

   1X TAE buffer was prepared by diluting 50X TAE buffer 50 times with double distilled water. pH of the final buffer will be ~8.6. Composition of 1X TAE buffer is 40 mM Tris base, 20 mM Acetate and 1 mM EDTA.

2. **1.2% Agarose gel**

   Agarose gel was prepared by dispersing agarose powder (1.2 g) in 100 mL of 1X TAE buffer. Dispersed agarose is heated at 90 °C with intermittent shanking to completely dissolve the agarose powder in buffer. Agarose solution is allowed to cool to a consistency that can be easily poured and then poured in a gel tray tightly sealed in the casting tray up to 4-6 mm. A comb is placed in the gel to create wells for loading pDNA samples. The gel was allowed to completely set for 30-40 minutes at room temperature. Combs were removed appropriately without distorting the wells.

3. **EtBr stock solution (10 mg/mL)**

   Ethidium Bromide (EtBr) stock solution was prepared by dissolving EtBr powder in double distilled water. EtBr was used as an intercalating/staining dye which intercalates into the major grove of the DNA and fluoresces in UV light. In presence of DNA its fluorescence increases 25 times.

4. **Tank buffer:**

   Tank buffer was prepared by adding EtBr stock solution in the 1X TAE buffer at level of 0.5 µg/mL concentration. (Final level of EtBr in Tank buffer 0.5 µg/mL)

5. Loading buffer:

Gel loading buffer (6X) was purchased from HiMedia Labs. 6X loading buffer was used at 2 µL per well for loading pDNA samples. Gel loading buffer included bromphenol blue as a tracking dye to monitor the run of the electrophoresis and sucrose to raise the density of the sample so that sample sinks to the bottom of the well.

3.2.2.2 Method

Gel electrophoresis tank (GeNei Mini Sub System, Merck-Millipore-GeNei Techware, Banglore, India) was filled with tank buffer and electrodes were placed in tank. Electrodes were connected with voltage supplier (GeNei Electrophoresis Power Supplies, Merck-Millipore-GeNei Techware, Banglore, India). 1.2% agarose gel in gel tray was placed in the tank buffer immersing the gel 2-3 mm below the level of buffer with gel-end having wells towards the negative electrode and other end of gel towards the positive electrode. Each DNA sample was mixed with 3µL of 6X loading dye and then loaded on to gel. Electrophoresis run was carried out at 5 V/cm depending on the distance between the electrodes for 45 min to 60 min. Gel was removed after run and pDNA migrated on the gel was visualized under UV light at 254 nm on GelDoc™ Imaging System (Bio-Rad, USA). The gel was removed and the pDNA in the agarose gel was visualized under UV light using GelDoc™ XR+ Imaging System (Bio-Rad, USA). Gel images were taken on ImageLab Software (Version 4.0 Build 16, Bio-Ra Laboratories, USA). Gel images were analyzed by ImageJ software (ImageJ Ver. 1.49, NIH, USA) for quantification. For quantification, relative band densities were determined by ratio of area under curve (AUC) of bands against AUC of band of 200 ng pDNA (i.e. considering the band density of 200 ng to be 1).

3.2.2.3. Results and Discussion

a) Determination of detectable range of DNA for Gel Retardation Assay

pDNA was loaded in a fixed volume of 20 µL but at different concentration (25, 50 75, 100, 200, 300, 400 and 500 ng per well) along with the loading buffer and the EtBr on agarose gel and electrophoresis was performed to determine the quantifiable range of pDNA. Purpose of the experiment was to determine concentration of pDNA that can be quantified using gel electrophoresis. After run, gel was removed and migrated pDNA was visualized under UV light using GelDoc™ XR+ Imaging System (BioRad, USA). UV-

visualized gel (**Figure 3.4 Determination of quantifiable range of pDNA**) showed that ≥ 50 ng concentrations of pDNA are detectable.

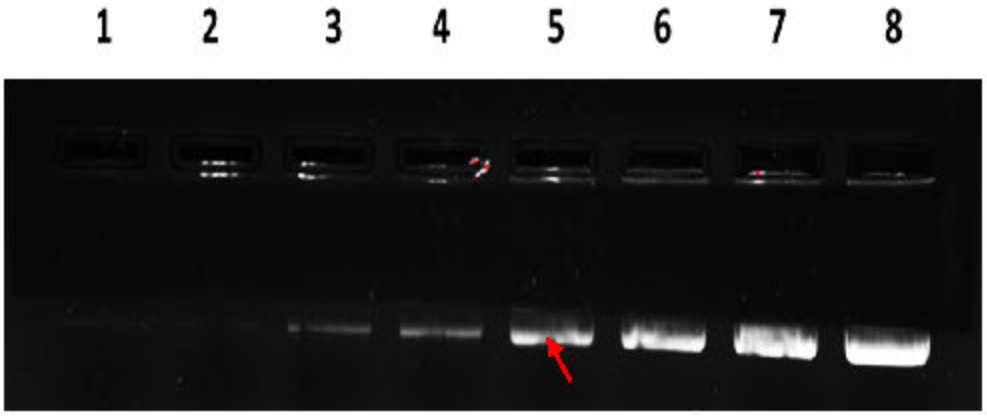

Figure 3.4 Determination of quantifiable range of pDNA (pDNA/well, Lane 1-10 ng, lane 2-20 ng, lane 3-50 ng, lane 4-100 ng, lane 5-200 ng, lane 6-300 ng, lane 7-400 ng, lane 8-500 ng)

b) Relative Quantification

200 ng band was evaluated by repeated analysis by using band densitometry to see its appropriateness to act as a reference band for relative quantification. Gel was loaded with 200 ng pDNA concentrations in 6 wells. Relative band densities were determined using one band as a reference band (**Figure 3.5** and **Table 3.6**). Relative band densities were found to be in agreement within 3% RSD indicating the accuracy and precision of the method. Percentage recovery of the pDNA was found to be 203.66 ng±5.63 ng.

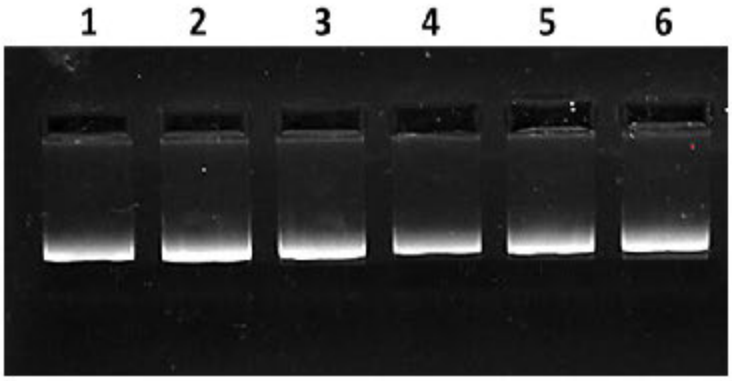

Figure 3.5 Band densities at 200 ng Lane 1 to Lane 6: 200 ng pDNA.

Table 3.6 Relative band density of 200 ng pDNA

Band No	Relative and Density	pDNA recovery (ng)
1	0.998	199.711
2	0.986	196.722
3	1.047	211.235
4	1.031	205.453
5	1.026	205.196
Mean	1.018	203.663
SD	0.025	5.628
%RSD	2.456	2.763

Calibration curve for relative quantification of pDNA was constructed by taking the band density at 200 ng concentration as 1.00 and evaluating other band densities relative to this concentration. Briefly, pDNA solutions of different concentrations (10-200 ng) were prepared and mixed with gel loading buffer and gel electrophoresis was performed as described above. Analysis was repeated three times and measurement error (as standard deviation) was calculated. **Table 3.7** shows band densities obtained with aforesaid pDNA concentrations as well as their relative band densities as compared to 200 ng concentration.

Calibration curve (**Figure 3.6**) of observed concentrations of pDNA against taken concentrations was generated using values depicted in **Table 3.7**. The method will be accurate with a maximum deviation of ~5% of the present level of pDNA. This would allow for fast determination of complexed pDNA with sufficient accuracy and precision to make the appropriate conclusions. The determination will be further confirmed by UV spectrophotometric and spectrofluorometric assays for selected formulations which are found to be more accurate and precise methods.

Table 3.7 Relative band densities at different pDNA concentrations

pDNA taken (ng)	Relative band densities		pDNA observed (ng)		%RSD
	Mean	SD	Mean	SD	
200	1.000	-	-	-	
100	0.488	0.011	98.50	2.411	2.448
50	0.242	0.010	49.820	1.760	3.533
40	0.198	0.009	39.002	1.409	3.613
20	0.089	0.006	18.186	0.832	4.575

10	0.046	0.003	8.993	0.468	5.204

*Values are represented as mean±SD, n=3.

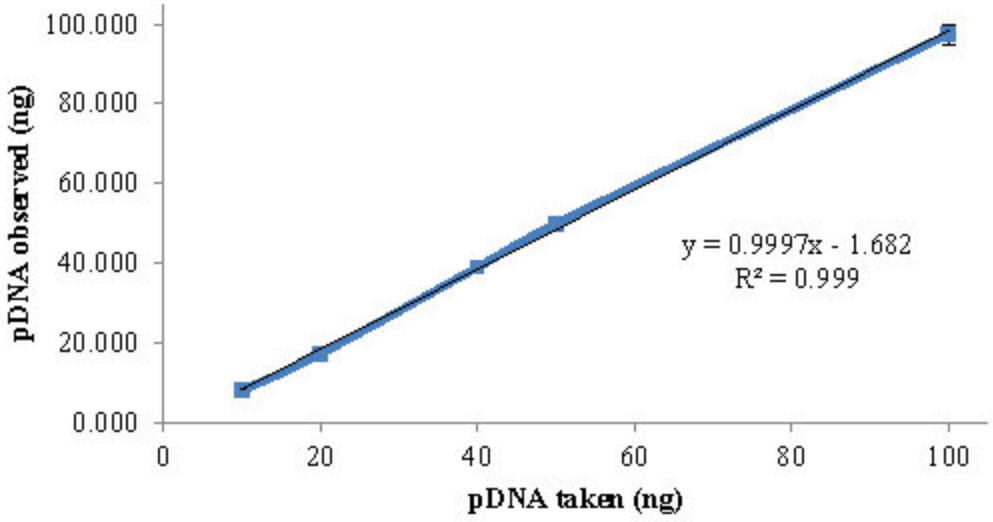

Figure 3.6 Calibration plot of DNA gel retardation.

% Recovery and % Relative Standard Deviation of the method were found to be 99.79±0.39% and 1.4% respectively which depict the accuracy and reproducibility of the method respectively. Therefore, the proposed analytical method for quantification of pDNA was found to be reliable for routine estimations.

3.3 Analytical methods used for characterization of lipids

Analytical methods used in the synthesis of lipids were TNBS assay (which was used to detect free unconjugated lipids) and Sakaguchi assay (which was used to determine quantitate guanidine groups) in order to determine the conjugation efficiency of the synthesis method employed for conjugation of lipid with Boc-amino acid derivatives.

3.3.1 TNBS assay

2,4,6-Trinitrobenzene Sulfonic Acid (TNBSA or TNBS) is a rapid and sensitive assay reagent for the determination of free amino groups. Primary amines, upon reaction with TNBSA, form a highly chromogenic derivative, which can be measured at 340 nm (**Figure 3.7**). Qualitative measurements of amines, sulfhydryls or hydrazides, and quantitative measurements of ∑- amino groups of L-lysine have also been obtained using TNBSA. It is supplied as a 1% solution in methanol.

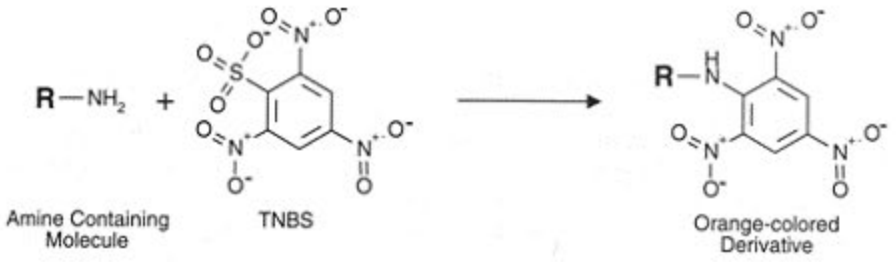

Figure 3.7 Reaction of primary amine containing compound with TNBS

3.3.1.1 Materials

1. Solvent: Solvent for TNBS reaction was prepared by mixing 5:4:1 v/v/v ratio of chloroform, methanol and water. pH of the solvent mixture was adjusted to approx. 8.5 using 0.01% triethyl amine and measured using pH strip.
2. TNBS solution: Available as 5%v/v solution in methanol from Thermo scientific (USA). The concentration was made to 0.01% TNBS solution using above prepared solvent.
3. 10% sodium dodecyl sulfate solution: 10 g of sodium dodecyl sulfate (SDS) was dissolved in water and volume was made up to 100 mL.
4. 1 N HCl solution: 0.85 mL of concentrated HCl was added to 50 mL water in a 100mL volumetric flask and then diluted to 100 mL.

3.3.1.2 Method

The solvent system was modified for the analysis and consisted of Chloroform: Methanol: Water in the ratio of 5: 4: 1 by volume adjusted to pH 8.5 using 0.01% triethyl amine. The solvent mixture was chosen based on the solubility of the lipids and to provide reaction specific condition (pH 8.5) without affecting the reaction between the TNBS and amine. The solutions of stearyl amine (5 ppm to 25 ppm) and DSPE (5 ppm to 25 ppm) separately were prepared in the solvent mixture. 0.25 ml of the 0.01% (w/v) solution of TNBSA (Thermo Scientific, USA) to 0.5 ml of each sample solution and mixed well. The solutions were incubated at 37°C for 1 hr, 3 hr and 6 hr. 0.25 ml of 10% SDS and 0.125 ml of 1 N HCl were added to each sample after incubation and absorption spectra of solutions were recorded in the range of 200 nm to 600 nm on UV 1800 spectrophotometer

(Shimadzu, Japan). Blank reaction mixture (solvent mixture without any lipid treated the same way with TNBS as the solution of lipid) was used as a reference to nullify any absorbance by reagent blank. TNBS over time produces yellow discoloration which can impact the results, hence, blank results will negate the effect the absorbance by the reagent blank. Calibration curves were developed for all lipids (Stearyl amine and DSPE) for absorbance at 340 nm and 415 nm.

3.3.1.3 Results and discussion

Calibration curves were developed for stearyl amine and DSPE using TNBS assay **Table 3.8**. The calibration curves will help detect the unreacted amines in the reaction after conjugation with targeting ligand (galactose) & also for detecting DSPE which has been used to conjugate with Boc-histidine and Boc-carnosine. As conjugated lipids will have no primary amine to react with TNBS, only unconjugated lipids from the mixture will take part in the reaction giving the conjugation efficiency of the reaction. Additionally, using molecular weight, conjugation efficiency by weight can be transformed into the molar conjugation efficiency.

Overlay spectra of TNBS assay of stearyl amine over range of 5 ppm to 25 ppm carried out for 1 hr incubation period is shown in **Figure 3.8** and calibration curves at 340 nm and 415 nm are shown in **Figure 3.9**. Absorption values showed somewhat negative deviation at higher concentrations i.e. ≥20 ppm, hence, calibration curve was developed at concentrations of 2.5 ppm to 15 ppm. Due to higher response rate on 340 nm absorbance values of reactant product, calibration curve generated at 340 nm was considered for analysis. As the reaction solvent was changed from recommended (aqueous reaction mixture) to organic reaction solvent (Chloroform:methanol:water), the reaction time required for better analyses was of 1 hr and was used for subsequent analyses (as no change in readings was observed at the end of 3 hr and 6 hr incubation period - data not shown). Standard deviation error bars are shown in calibration curves but are not visible due to marker points on the curves. The absorbance values followed linear relation with a straight-line $y = 0.0569x + 0.0241$ with $R^2 = 0.9989$ at 340 nm.

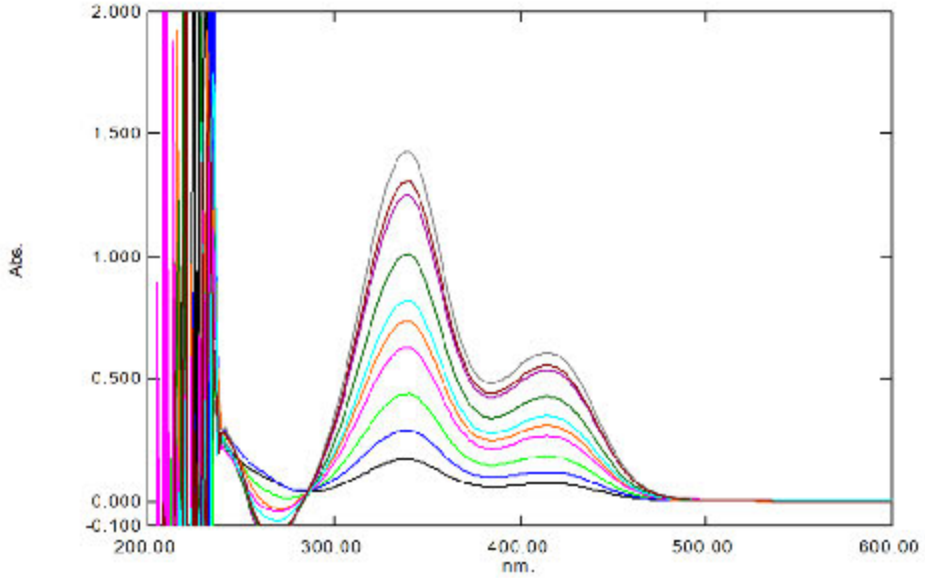

Figure 3.8 Overlay spectra of TNBS assay for stearyl amine

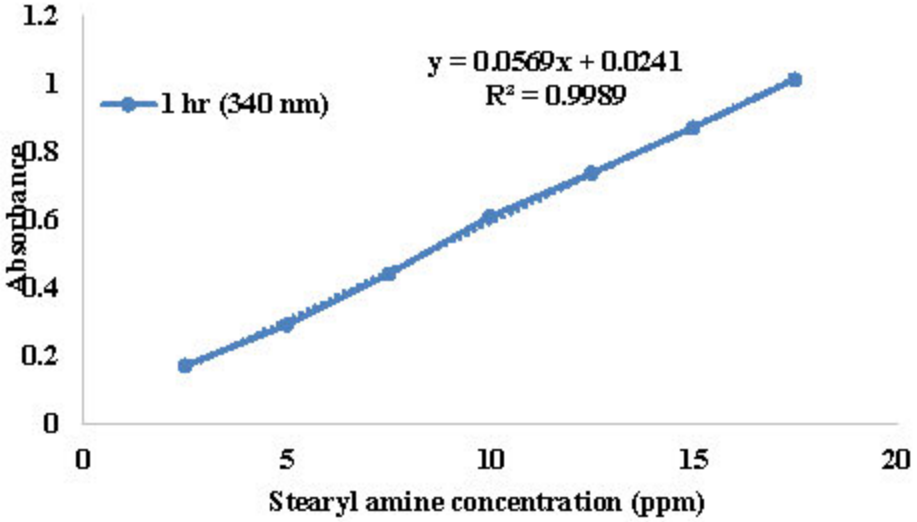

Figure 3.9 Calibration curve of TNBS assay of stearyl amine

Table 3.8 Mean absorbance values of TNBS assay carried out at incubation period of 1 hr.

Concentration of Stearylamine (ppm)	Mean absorbance values at 340 nm after incubation period of 1 hr
2.5	0.173
5	0.292
7.5	0.441
10	0.610
12.5	0.736
15	0.871

17.5	1.011
20	1.251
22.5	1.309
25	1.427

Accuracy and precision in terms of percentage recovery and percentage relative standard deviation (%RSD) respectively were evaluated for TNBS assay of stearyl amine. Solution of 2.5 ppm, 12.5 ppm and 25 ppm of stearyl amine were analysed by TNBS assay and % recovery was calculated. The reproducibility of the method was determined by analyses of samples at different time points to account for intraday and interday variability and determining the %RSD. **Table 3.9** and **Table 3.10**. represent accuracy, intraday and interday precision of the method, respectively. As it can be seen, the % recovery was found to be >99 % and the precision data had % RSD values were less than 3%.

Table 3.9 Accuracy of the TNBS assay of stearyl amine

Actual Concentration of SA (ppm)	Observed Concentration* (ppm)	Standard Deviation* (SD)	%Recovery
2.50	2.48	0.07	99.20
12.50	12.42	0.20	99.36
25.00	24.81	0.36	99.24

*Values are represented as mean±SD, n=3.

Table 3.10 Interday and intraday precision of the TNBS assay of stearyl amine

Actual Concentration (ppm)	Intraday precision			Interday precision		
	Observed Concentration* (ppm)	SD* (ppm)	%RSD	Observed Concentration* (ppm)	SD* (ppm)	%RSD
2.50	2.47	0.07	2.83	2.48	0.07	2.82
12.50	12.48	0.23	1.84	12.45	0.23	1.85
25.00	24.79	0.40	1.61	24.80	0.41	1.65

*Values are represented as mean ± SD, n=3.

Construction of DSPE calibration curve in the range of 5 to 25 ppm (**Figure 3.10 & figure 3.11**) was done. The absorbance values followed linear relation with a straight-line y = 0.0315x + 0.0080 and $R^2 = 0.9979$.

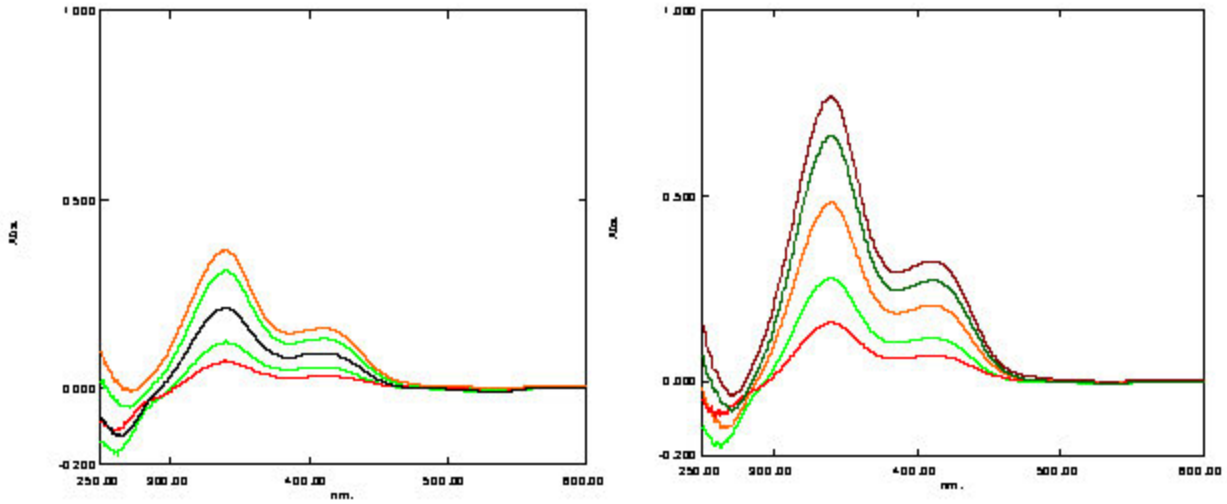

Figure 3.10 Overlay spectra of TNBS assay of DSPE A) Left pane→1 hr reaction time B) Right panel→ 6 hr reaction time

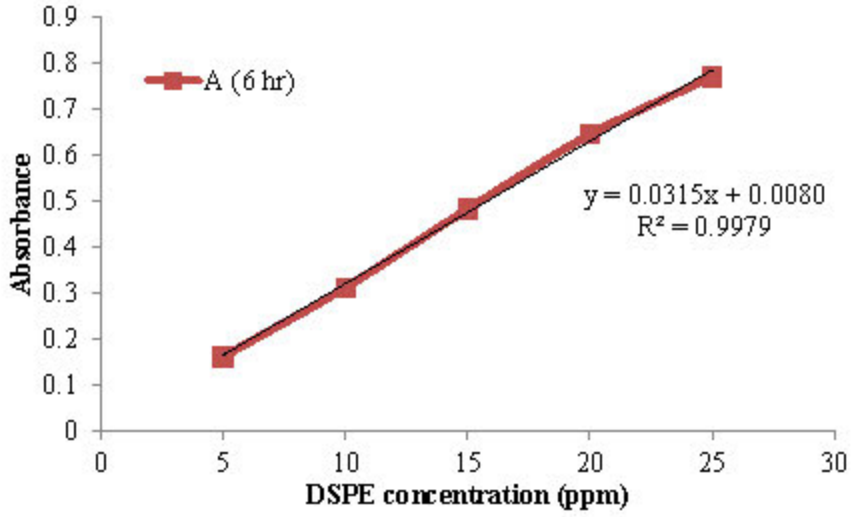

Figure 3.11 Calibration curve of TNBS assay of DSPE at 340 nm

Accuracy and precision of the method were determined for TNBS assay of DSPE. **Table 3.11** and **Table 3.12** represent accuracy, intraday and interday precision of the method, respectively. As it can be seen, the % recovery was found to be >99.0% and the % RSD values were less than 4%.

Table 3.11 Accuracy of the TNBS assay of DSPE

Actual Concentration of SA (ppm)	Observed Concentration* (ppm)	Standard Deviation* (SD)	%Recovery
5.00	4.97	0.15	99.40
12.50	12.41	0.26	99.28
25.00	24.83	0.41	99.32

*Values are represented as mean±SD, n=3.

Table 3.12 Intraday and interday precision of the TNBS assay of DSPE

Actual Concentration (ppm)	Intraday precision			Interday precision		
	Observed Concentration* (ppm)	SD* (ppm)	%RSD	Observed Concentration* (ppm)	SD* (ppm)	%RSD
5.00	4.96	0.14	2.82	4.86	0.19	3.91
12.50	12.40	0.25	2.01	12.38	0.36	2.91
25.00	24.89	0.36	1.45	24.75	0.56	2.26

*Values are represented as mean ± SD, n=3.

3.3.2 Sakaguchi assay

Sakaguchi reaction is a precise method used to estimate the guanidine residues in an amino acid. Hence, the reaction becomes important for estimation of arginine in proteins. The method will also be useful for development of conjugation of arginine to other molecules to determine the conjugation efficiency. The reaction occurs between the guanidine and α-naphthol in the presence of alkaline condition. The reaction produces a reddish colour solution that shows absorbance maxima at 524 nm.

3.3.2.1 Materials

1. 10% v/v methanol: 10 mL of methanol was made up to 100 mL with distilled water in a volumetric flask.
2. 0.1% w/v α-naphthol: 100 mg of α-naphthol was dissolved in water in 100 mL volumetric flask and volume was made up to 100 mL with water.
3. Hypobromite solution: To solution of 20 g of NaOH in 75 ml water, 5 ml of bromine was added and the volume was made to 100 ml.
4. 40% urea solution: 4 g of urea was dissolved in water in a 10 mL volumetric flask and volume was made up to 10 mL.

3.3.2.2 Method

Stock solution of Boc-arginine (2 mg/ml) was prepared in methanol. Dilution were prepared with methanol to yield concentrations of 6.25 ppm, 12.5 ppm, 25 ppm, 50 ppm, 75 ppm, 100 ppm, 125 ppm ,150 ppm and 175 ppm (concentration of guanidine ranges from 20 – 564 µM). To the standard solutions, 0.1 ml of 10% methanol and 0.1 ml of 0.1% α-Naphthol were added and mixed. The solution was allowed to cool on ice for 15 min. To the cooled solution, 0.1 ml of freshly prepared hypobromite solution (5% v/v) was added. The above mixture was quickly mixed for 10 seconds and immediately equal volume of 40% urea solution was added to it. The UV spectra of solutions were recorded immediately after 30 seconds on UV 1800 spectrophotometer (Shimadzu, Japan).

3.3.2.3 Results and discussion

Method for estimation of guanidine group was developed in methanol as the Boc-arginine and Boc-arginine modified stearyl amine were soluble in methanol. Overlaid spectra of Sakaguchi assay of Boc-arginine is show in **Figure 3.12**. An absorption maximum was observed at 524 nm. It was noted that at lower concentrations, linearity was not observed (slightly negative deviation was observed). Hence, calibration curve was plotted at concentrations of 80-564 µM (**Figure 3.13**) at λ_{max} of 524 nm. Absorbance values followed linear relation with a straight-line $y = 0.0038x - 0.1480$ and $R^2 = 0.9978$.

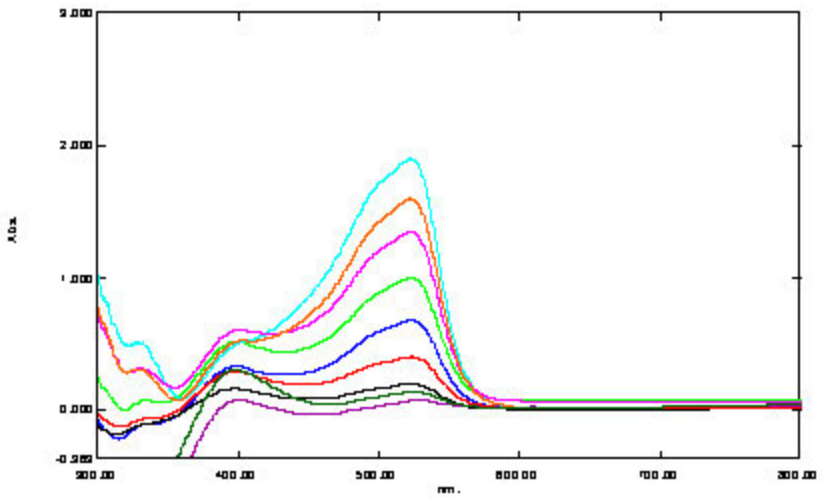

Figure 3.12 Overlay spectra of Sakaguchi assay of Boc-arginine (X-axis: Absorbance, Y-axis: wavelength (nm))

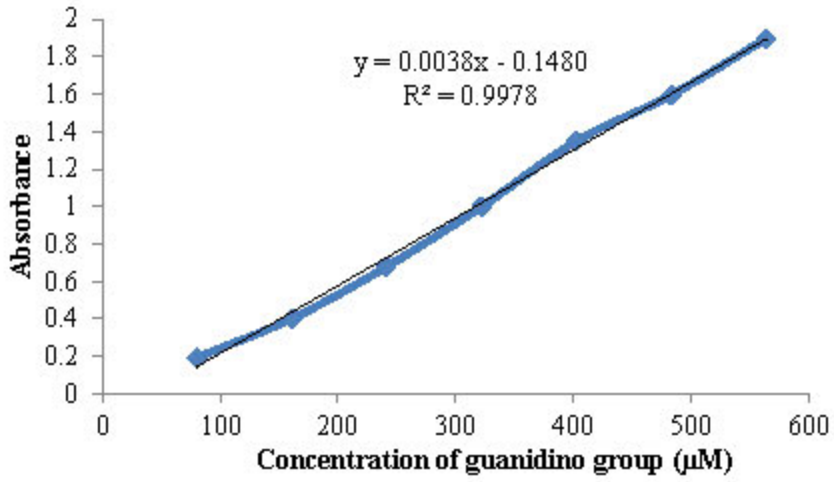

Figure 3.13 Calibration curve of Sakaguchi assay of Boc-arginine

Accuracy and precision in terms of percentage recovery and percentage relative standard deviation (%RSD) respectively were evaluated for Sakaguchi assay. Solution of 81 µM, 322 µM and 564 µM concentrations of Boc-arginine were analysed by Sakaguchi assay and % recovery was calculated. The reproducibility of the method was determined by the analyses of samples at different time points to account for intraday and interday variability and determining the %RSD.

Table 3.13 and **Table 3.14** represent accuracy, intraday and interday precision of the method, respectively. As it can be seen, the % recovery was found to be >98.0% and the precision data indicate % RSD values were less than 4%.

Table 3.13 Accuracy of the Sakaguchi assay of Boc-arginine

Actual Concentration of SA (ppm)	Observed Concentration* (ppm)	Standard Deviation* (SD)	%Recovery
80.0	78.99	1.51	98.74
322.0	317.81	3.36	98.70
564.0	560.20	4.87	99.32

*Values are represented as mean±SD, n=3.

Table 3.14 Intraday and interday precision of Sakaguchi assay of Boc-arginine

Actual Concentration (ppm)	Intraday precision			Interday precision		
	Observed Concentration* (ppm)	SD* (ppm)	%RSD	Observed Concentration* (ppm)	SD* (ppm)	%RSD
80.0	77.24	2.79	3.61	78.96	3.05	3.87
322.0	319.21	8.15	2.55	318.47	7.15	2.24
564.0	560.28	10.94	1.95	558.49	11.55	2.07

*Values are represented as mean ± SD, n=3.

3.4 References

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