3. Introduction

For the successful development any formulation the analytical method should be established for the quantification of active pharmaceutical ingredient (API) involved. Same is the necessity in the case of nucleic acid, an effective method should be developed for the quantification of entrapped DNA in formulated lipoplex formulation and other stability indicating studies.

3.1 DNA quantification by UV spectrometry

It is one of the very simple, fast and non-destructive type of method for DNA quantification. The nucleic acid absorption pattern also follows the Beer-Lambert Law which represents the linear relation between concentration and absorption (1). Nucleotides present in DNA sample has tendency to absorb UV light which can be used as a method to estimate and quantify DNA present in our sample. If the DNA is in pure form in our sample than i.e. devoid of protein contamination and organic solvents used in isolation process; the sample shows maximum absorption around 260 nm owing to presence of purines and pyrimidines in DNA or nucleic acid e.g. absorption maxima for dATP, dCTP and dTTP is 259 mm, 272 nm and 247 nm respectively (2). During the estimation of total plasmid DNA present is sample, sheared DNA, aromatic amino acids and presence of RNA can hamper with the accurate estimation of DNA. Aromatic amino acid present in sample absorb maximum UV light at around 280 nm (3). In the same way, absorption at 230 nm represents the quantity of RNA present in sample. Absorption reading at 320 nm which is indication of light scattering components if any present in our sample is subtracted from the absorption reading at 280 nm as well as 260 nm as background reading and then after the ratio of newly obtained absorption value at 260 nm and 280 nm should be used to check for the purity of DNA present in sample. This absorption spectroscopy method is however limited by its insensitivity which requires at least 1 µg/mL quantity of DNA. Chances of absorption to be hampered by high amount of RNA presence is also a big drawback of this method. In short, accurate analysis of the DNA preparation may be impeded by the presence of impurities in the sample or if the amount of DNA is too little. To justify this necessity, nanodrop instrument and gel electrophoresis methods were used to estimate purity of DNA sample and estimation of larger quantity of sample in minute concentration as well as in high concentration (4).

3.2 Nanodrop spectrophotometer

The usual application is quite easier even though the working principle is same. The law of the Beer-Lambert demonstrates the direct relation between concentration of solute in

solvent and its absorbance. Because of the presence of aromatic base in their structures, nucleic acids have peak of UB absorbance at 260 nm while they have ability to absorb UV lights at more than one wavelengths. Thus, absorbance at 260 nm can be used to seek concentration of nucleic acid in solution at the expanse of light absorbance at 260 nm using Beer-Lambert law. But this law can be applied only for the absorbance ranging from 0.1 to 1.0. This converts the concentration of nucleic acid in range between 10.0 ng/µL and 3700 ng/µL using the Nanodrop ND-1000. Concentration outside this range should be further diluted for more accurate results. Figure 3.1 shows the working principle of nanodrop spectrophotometer.

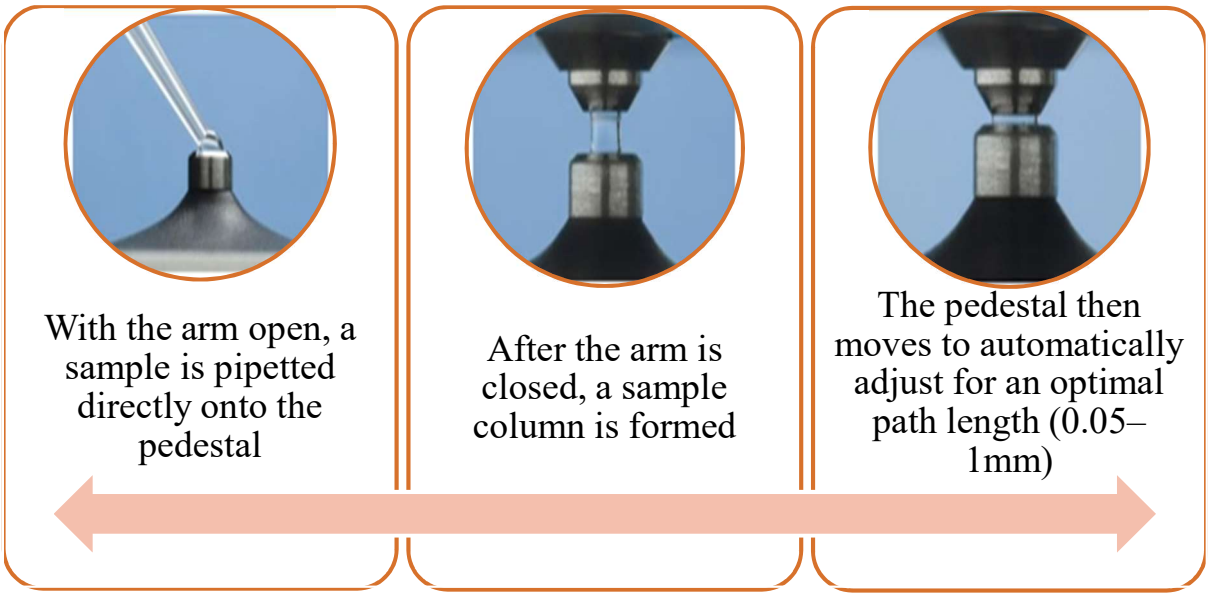

Figure 3. 1 Working of NanoDrop instrument for the assessment of cDNA concentration

Preparation of DNAse Free Water (DFW)

0.1 % of DEPC water mixture was prepared by adding 1 mL of Diethyl Polycarbonate (DEPC) to the 1 L of distilled water and overnight stirred using magnetic stirrer. Prepared mixture was then autoclaved at 121 ℃, 15 Psi for around 15-20 minutes and filtered through 0.2 µm sized syringe filter to prepare DFW. This prepared DFW was further used for any preparation or analysis involving cDNA (5).

3.2.1 Method verification

Method verification was performed by preparing appropriate dilutions of stock solution with DFW. Then cDNA dilutions of various concentrations were prepared using DWF. Absorbance of the prepared solutions were measured using a nanoDrop UV spectrophotometer and the method.

3.3 Gel electrophoresis for cDNA quantification

Gel electrophoresis has become a mainstream technique for the analysis or quantification of cDNA in comparison to colorimetry and spectrophotometric analysis which provide only quantitative assessment of cDNA (6). A picture taken after gel development provides information regarding to its quantity as well as quality of cDNA and RNA contamination. With the easy availability of hardware to run gel and software for data accumulation, gel electrophoresis has become a method of choice for many researchers. Agarose gel electrophoresis is the easiest and commonest way of separating and analyzing DNA. The purpose of the gel might be to look at the DNA, to quantify it or to isolate a particular band. Most agarose gels are made between 0.7% and 2%. A 0.7% gel will show good separation (resolution) of large DNA fragments (5–10 kb) and a 2% gel will show good resolution for small fragments (0.2–1 kb) (7). Some people go as high as 3% for separating very tiny fragments but a vertical polyacrylamide gel is more appropriate in this case. Low percentage gels are very weak and may break when you try to lift them. High percentage gels are often brittle and do not set evenly. Figure 3.2 shows the arrangement of agarose gel electrophoresis unit.

The most prominent and frequently used dye for the detection of nucleic acid is ethidium bromide. It works with DNA interchelation, introducing itself into the places available between the double helical base pairs of DNA. Ethidium bromide absorbs the UV light maximum at 300 and 360 nm. Moreover, the energy can also be absorbed from nucleotide excitation from the 260 nm wavelength. Ethidium has ability to re-emit the energy in form of yellow or orange light galloped at 590 nm (8). The ethidium bromide fluorescence with interchelated dye is much higher with respect to its solution form. In this way, it is one the most sensitive, precise and easy to use dye/stain for DNA. It has capability to generate low background with the detection limit around 1-5 ng/band. But the carcinogenic potential associated with its use is the main disadvantage linked to the use of ethidium bromide. It needs to be handled with great care and needs to be purified before disposing or removing it. However, its sensitivity towards nucleic acid, simple is use and non-destructive nature have put it in the standard list of stains or dyes used for the double stranded DNA (dsDNA).

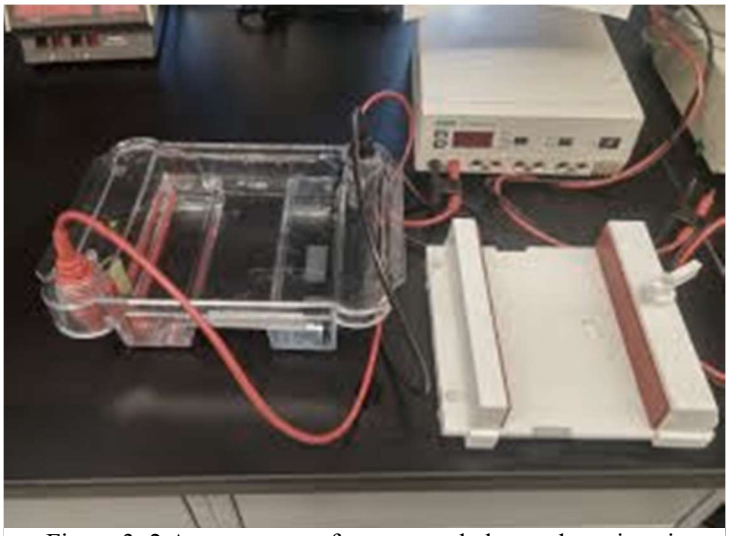

Figure 3. 2 Arrangement of agarose gel electrophoresis unit

Protocol

3.4 Analytical methods for lipid characterization

TNBS assay was used to detect quantity of DOPE (lipid) in its unconjugated form through identification of free amine group present on lipid DOPE. In a way, this assay was used to determine the conjugation efficiency of DOPE to BOC protected lipids.

3.4.1 TNBS assay

TNBSA/TNBS or 2, 4, 6-Trinitrobenzene Sulfonic Acid is a quick and subtle assay reagent for the assessment of free amino groups. Primary amines, when reacted with TNBSA, create a very chromogenic substance, which can be easily measured at 340 nm using spectrophotometer. Qualitative assessment of primary amines, hydrazides or sulfhydryl, and accurate quantitative determination of ∑- amino groups present in L-lysine can also be properly obtained using TNBSA (9). TNBS solution is supplied as 1 % solution in methanol.

Procedure

The solvent system for the use of TNBSA for the analysis of primary amines involved Chloroform: Methanol: Water in the ratio of 5: 4: 1 along with 0.8 M sodium bicarbonate (pH 8.5). The solvent mixture preferred, was based on the solubility of the DOPE and to arrange for reaction specific condition (pH 8.5) without upsetting the reaction between the TNBS and primary amine. Solutions of DOPE ranging from 5 ppm to 25 ppm were prepared in the solvent mixture as depicted above. To these prepared solutions, 0.01 % w/v TNBSA solution was added in half quantity to the original solution quantity and mixed well. 0.50 mL of 10 % SDS and 0.250 mL of 1 N HCl was added to each solution mixture after

incubating for 1 h at 37°C and the spectra was recorded for 200 to 600 nm range on UV 1800 spectrophotometer (Shimadzu, Japan). Blank reaction mixture was used as a reference to nullify any interference created with TNBSA absorbance. TNBS solution overtime produces yellow to orange coloration which may affect the result integrity therefore absorbance from the blank solution can negate this effects. Calibration curve was developed at 340 nm using UV spectrophotometer.

3.5 Bradfords protein assay

For any biochemical practice, assessment of protein concentration is one of the most important everyday task. Many method can be used for this purpose to suit our criteria. There is not only one reason to find out the concentration of protein on daily basis in work related to biochemistry. Evaluation of enzymatic activity against the concentration of protein is important for the purity of sample during the process of protein purification. Another application would be during analysis using SDS-PAGE or during the usage of antibody with western blot application, it is a necessity to add same amount of protein to achieve similar results. Reaction between dye and protein of interest will result in complex that has tendency to absorb particular wavelength. This Bradford assay is commonly known as “The Coomassie brilliant blue protein assay” which is relatively rapid and follows very simple protocol (10). Prior to addition of protein in solution, brafords reagent would appear as reddish brown in colour while after addition it turns into blue colour owing to pka shift of dye which can be analysed at 595 nm.

Procedure:

Stock solution of 0.1 µg/µL concentrated standard bovine serum solution was prepared. Any other standard can be used for the same, provided the same reagent should be used throughout the experiments. With proper dilution using deionised water, concentration range of 1-150 µg/µL was attained. Concentrations higher or lower than this range can also be used as there is no limitation in achieving linearity range using particular concentration. In elisa plate reader, 100 µL of sample solution was added to each wells and Add 100 µl of the diluted Bradford reagent to all wells. 200 µl of deionised water sample is uses as blank; wait for at least 5 min after addition of Bradfords reagent to develop colour which was analysed at 595 nm using elisa plate reader.

3.6 Result and discussion

3.6.1 Nanodrop

Results in figure 3.3 shows that the isolated plasmid DNA fulfil the purity criteria as the ratio of A260/A280 and A260/A230 are within the standard acceptable range. The sample used for the analysis was around 10 µL which could be further diluted if cDNA quantity is out of measurable limits of nanodrop. The correlation between prepared and obtained dilution of plasmid DNA showed that the method can be used for the accurate determination of cDNA in wide concentration range as shown in table 3.1.

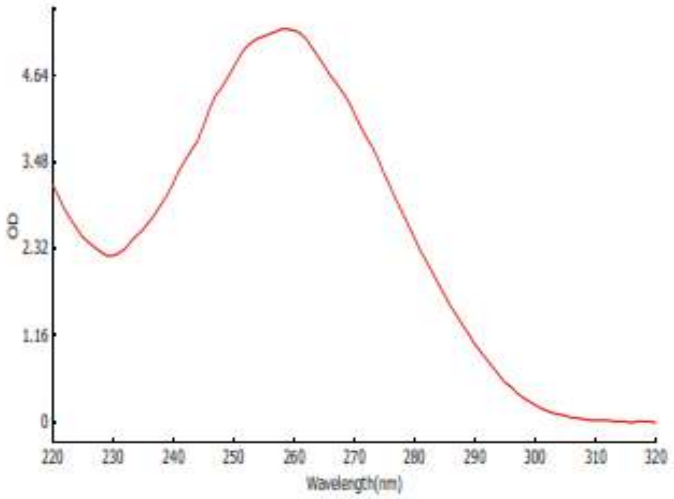

Figure 3. 3 Spectra of ssDNA (p11 cDNA) using NanoDrop

Table 3. 1 Purity and concentration of p11 cDNA using NanoDrop instrument

Item	Result
OD260	5.037
OD280	2.748
OD230	2.559
OD320	0.009
OD260/280	1.83
OD260/230	1.97
Nucleic acid concentration	262.63 ng/ µL
Pathlength (mm)	0.719
Dilution	1.00

Table 3. 2 Standard deviation between obtained and actual concentrations of cDNA solutions

Concentration of cDNA (ng/µL)	Obtained concentration (ng/ µL)	Standard deviation	% relative standard deviation
10.0	10.21	0.127	1.24
20.0	21.05	0.152	0.72
30.0	30.97	0.105	0.34
40.0	42.14	0.194	0.46
50.0	51.75	0.119	0.23

Values are represented as mean ± SD, n=3

Verification of method comprises of accuracy and precision of developed method as shown in table 3.2 and table 3.3. Accuracy is defined as a close correlation between observed and true calculated values for samples. Accuracy of samples were determined using repetitive measurements of three concentration of samples. Samples for this study were prepared from separated stock solution prepared using DFW and they were also analysed in triplicate. Accuracy was quantified using mean percentage recovery as deciding criteria using three different concentrations. Repeatability was measured using three different concentration as were used for accuracy studies, in inter-day and intraday variations where inter-day variation studies were carried out for three days.

As shown in table 3.3, recovery was found in range of 98.0 % to 102.0 % which was well within the acceptable limit. Moreover, % relative standard deviation (% RSD) was found to be below 2 %, for all the concentrations which is acceptable as per the ICH guidelines. Therefore, this method can be useful for the analysis of cDNA with accuracy.

Table 3. 3 Accuracy measurements of cDNA concentrations

Sample concentration (ng/µL)	Obtained concentration (ng/µL)	Standard deviation	% Recovery
10.0	10.14	0.194	101.4
30.0	29.89	0.083	99.6
50.0	50.81	0.171	101.6

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

Table 3. 4 Inter-day and intraday precision of the method

	Observed concentration		% relative standard deviation	
Concentration (ng/µL)	**Intraday precision (ng/µL)**	**Inter-day precision (ng/µL)**	**Inter-day precision**	**Intraday precision**
10	10.25	10.37	1.285	1.327
30	29.41	29.54	0.647	0.518
50	50.56	50.47	0.971	0.839

Values are represented as mean ± SD, n=3

3.6.2 Gel electrophoresis

Determination of detectable range of cDNA

To quantify the cDNA, 20 µL of cDNA sample of varied concentrations (5, 10, 15, 20, 25 and 30 ng) along with loading buffer and EtBr as dye was loaded in wells and the electrophoresis was carried out. The sole purpose of carrying out this experiment was to find out the minimum quantifiable quantity of cDNA using gel electrophoresis. After completion of experiment, the agarose gel was removed and the bands of different densities

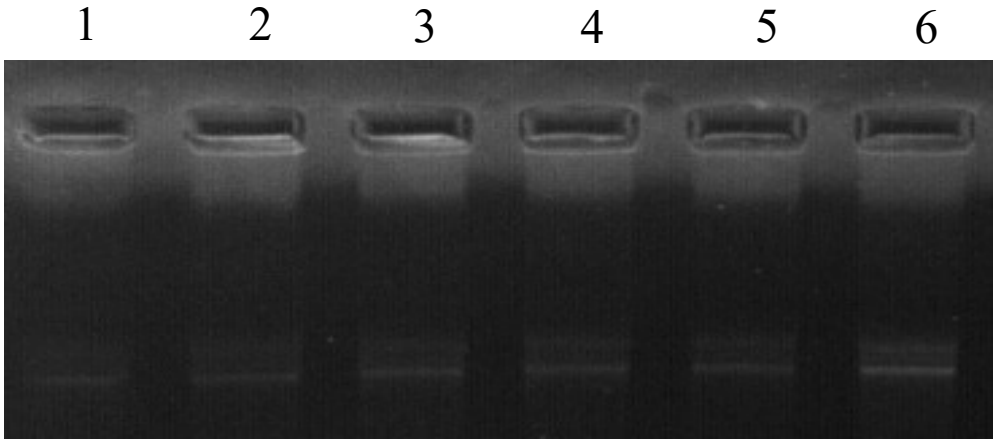

Figure 3. 4 Detection of quantifiable range of p11 cDNA

Lane 1- 5 ng, lane 2- 10 ng, lane 3- 15 ng, lane 4- 20 ng, lane 5- 25 ng, lane 6- 30 ng

were visualised using GelDoc™ XR $^{+}$ Imaging System (BioRad, USA). This study depicted that the cDNA in as low as 5 ng quantity can be detectable using gel electrophoresis as shown in figure 3.4 .

Calibration curve for cDNA quantification

Calibration curve for different concentration of cDNA was constructed using calculating the area of each band as a function of their density. Higher peak represents the darker bands

or in other word, as the concentration of cDNA sample in well increases, the density of the obtained band also increases resulting in larger peak area relative to other bands with less density. The plot as shown in figure 3.6 was constructed using this calculated area against the various concentrations ranging from 10 to 60 ng for the bands shown in figure 3.5. The obtained curve was found to follow the linear regression with r^2 value of 0.9989. Experiments were carried out in triplicate to confirm the accuracy of said method and to find out the deviation. This evaluation was further confirmed using nanodrop spectrophotometer which was found to be more accurate than this method.

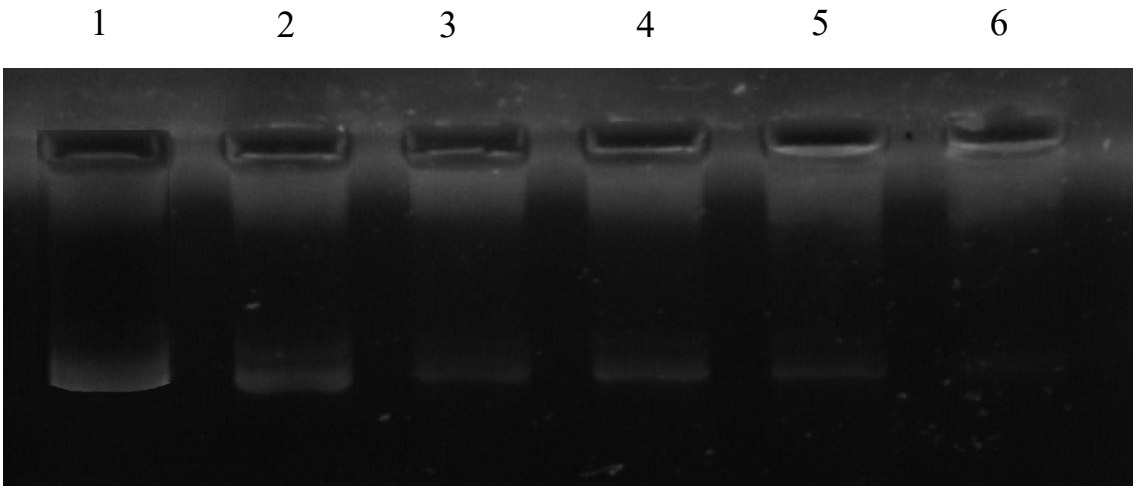

Figure 3. 5 Calibration curve of p11 cDNA using gel electrophoresis ranging from 10 ng to 60 ng

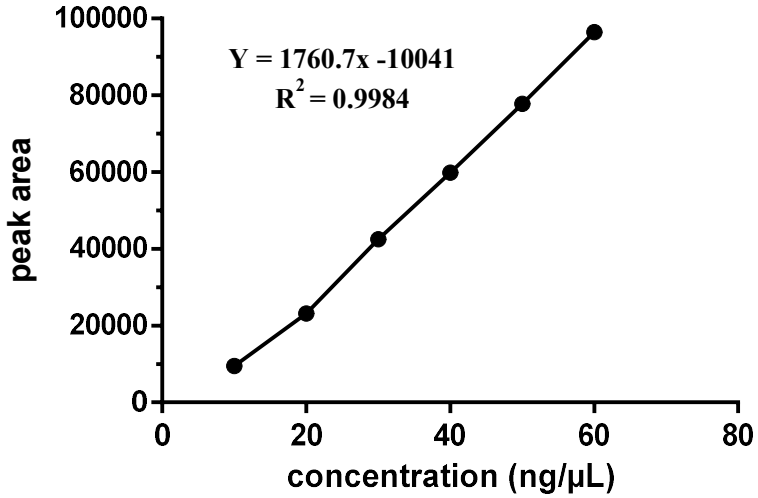

Figure 3. 6 The correlation curve of cDNA concentrations in ng/µL and peak area

Table 3. 5 Peak area and % RSD for actual and observed concentrations of cDNA

cDNA taken (ng)	Peak area		cDNA observed (ng)		% RSD
	Mean	SD	Mean	SD	
60	96497.81	1400.63	60.52	+ 0.52	0.86
50	77826.96	1237.92	49.91	- 0.09	0.18
40	59912.34	1001.61	39.73	- 0.27	0.67
30	42568.59	0787.48	29.87	- 0.13	0.43
20	23169.75	0712.33	18.86	- 1.14	6.04
10	9515.32	0459.76	11.11	+1.11	9.99

Values are represented as mean ± SD, n=3

Results from the table 3.5 also indicate that the method is more sensitive towards cDNA quantity higher than 20 ng as their % RSD value is in range. Below 30 ng the method is not suitable for accurate calculation of cDNA presence with their higher % RSD around 6.04 and 9.99 respectively for 20 ng and 10 ng.

3.6.3 TNBS assay

Calibration curves were developed for calculation of unreacted DOPE present in reaction mixture. The calibration curve can detect unreacted DOPE which has been used to conjugate BOC protected histidine, arginine and lysine. Conjugated lipids are devoid of primary amine which cannot react with TNBS to be detected using spectrophotometry. Only unreacted DOPE molecules which have primary amine in their structure that reacts with TNBS and UV spectroscopy helps in quantifying them at 345 nm. Overlay spectra of DOPE starting from 5 ppm to 20 ppm is shown in figure 3.7. The calibration curve is shown in figure 3.8 expressed by equation y= 0.0394 x + 0.1675 with correlation coefficient of 0.9988. The developed calibration curve was used further to find out conjugation efficiency of BOC protected amino acid to DOPE.

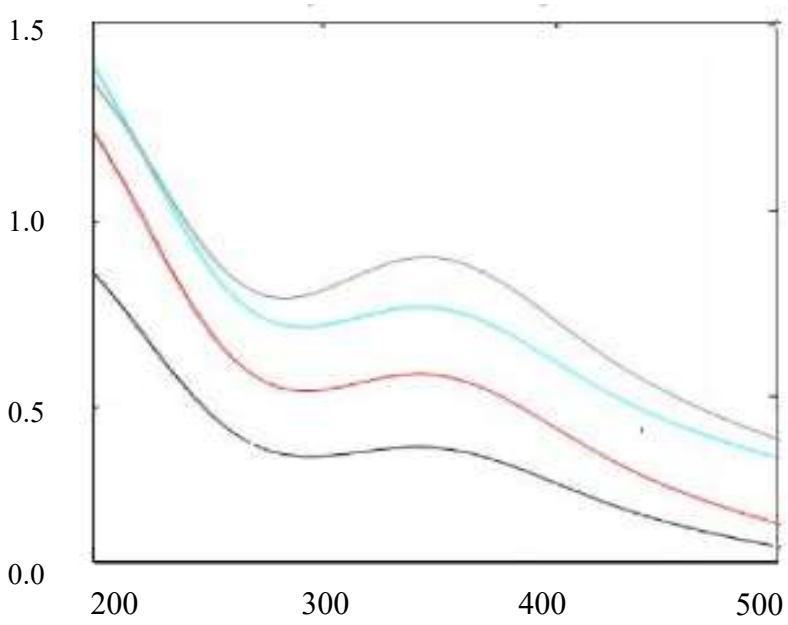

Figure 3. 7 Overlay spectra of TNBSA (TNBS) assay for DOPE

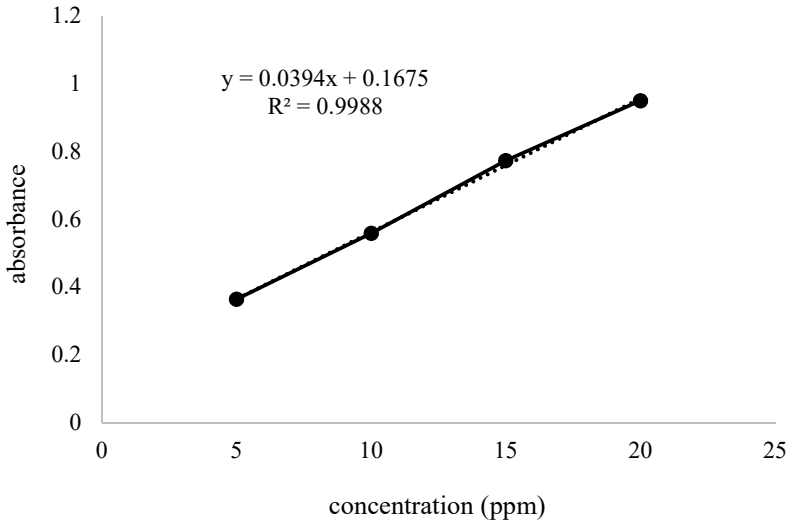

Figure 3. 8 The correlation spectra of TNBS assay for DOPE

3.6.4 Bradford assay

To estimate the protein content in cell lysate and brain tissue, Bradford method was used. In this method, bovine serum albumin was used as standard for the preparation of calibration. The formed complex of dye and protein was assessed at 595 nm and method was also found to be accurate and linear as shown in table 3.6 and figure 3.9.

Table 3. 6 Absorbance of BSA in linearity range from 1 to 150 µg/µL

Concentration (µg/µL)	Absorbance ± SD
1	0.487
10	0.525
25	0.572
50	0.643
75	0.719
150	0.916

Values are represented as mean ± SD, n=3

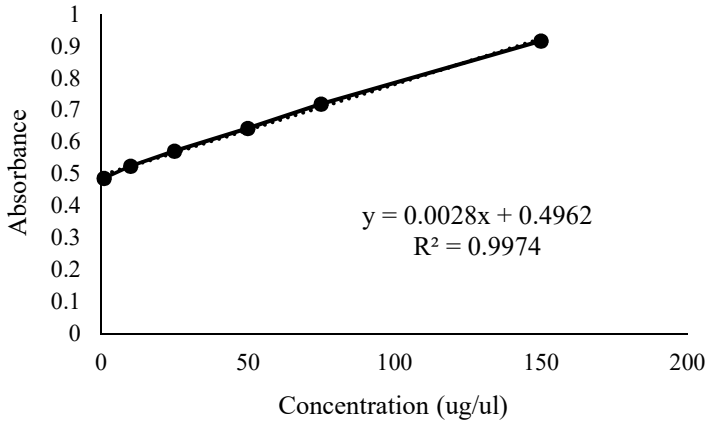

Figure 3. 9 Calibration curve for Bradford assay

References

1. Mach H, Volkin DB, Burke CJ, Middaugh CR. Ultraviolet absorption spectroscopy. Protein stability and folding: Springer; 1995. p. 91-114.
2. Reddy TP, Sahithi D. Amplification and Sequence Analysis of TPI Gene, a Structural Gene of Operon. Biotechnology and Bioforensics: New Trends. 2014.
3. Schmid FX. Biological Macromolecules: UV-visible Spectrophotometry. eLS. 2001.
4. Simbolo M, Gottardi M, Corbo V, Fassan M, Mafficini A, Malpeli G, et al. DNA qualification workflow for next generation sequencing of histopathological samples. PLoS One. 2013;8(6):e62692.
5. Greber-Platzer S, Schatzmann-Turhani D, Wollenek G, Lubec G. Evidence against the current hypothesis of "gene dosage effects" of trisomy 21: ets-2, encoded on chromosome 21" is not overexpressed in hearts of patients with Down syndrome. Biochemical and biophysical research communications. 1999;254(2):395-9.
6. Harris E. A low-cost approach to PCR: appropriate transfer of biomolecular techniques: Oxford University Press; 1998.
7. Smith CL, Cantor CR. Purification, specific fragmentation, and separation of large DNA molecules. Recombinant DNA Methodology: Elsevier; 1989. p. 139-57.
8. Foster KA, Galeffi F, Gerich FJ, Turner DA, Müller M. OPTICAL AND PHARMACOLOGICAL TOOLS TO INVESTIGATE THE ROLE OF MITOCHONDRIA DURING OXIDATIVE STRESS AND NEURODEGENERATION. Progress in neurobiology. 2006;79(3):136-71.
9. Traina CA, Bakus RC, 2nd, Bazan GC. Design and synthesis of monofunctionalized, water-soluble conjugated polymers for biosensing and imaging applications. Journal of the American Chemical Society. 2011;133(32):12600-7.
10. Ernst O, Zor T. Linearization of the bradford protein assay. Journal of visualized experiments: JoVE. 2010(38).