3. ANALYTICAL METHODS

3.1 INTRODUCTION

A validated analytical method is a crucial and fundamental component in formulation development. Analytical method required to estimate Cisplatin during preparation, optimization and characterization of HNCs was developed and partially validated for aptness of the analysis. The methods for analysis and estimation of Cisplatin at different stage such as entrapped drug estimation in the formulation, *In-vitro* drug release study at three different physiological conditions that exist in a cancer patient, the pH of the blood and lung tissue is 7.4, the pH at the cancer tissue is 6.6 and the pH inside the cancer cells range from 5-6, total phospholipids content and *In-vivo* pharmacokinetic study has been discussed in this chapter.

Analytical Method Validation

It is essential to authenticate the performance of an analytical method for acceptable estimation of an analyte. In following conditions, it is mandatory to validate analytical method; (1) when for a definite problem a new method is established; (2) when results of already established method are varying with time; (3) when a developed method is improved or extended for additional indication; (4) when the developed method is performed in a diverse laboratories by different instruments and analyst; (5) when the equality of a new and a conventional standard method has to be proved. As per the recommendation of various regulatory agencies accuracy, precision, linearity, selectivity, specificity, limit of detection, limit of quantification, robustness and ruggedness are suggested validation parameter (1, 2).

Accuracy

Accuracy expresses the closeness of estimated results to the standard or already accepted value. It is also referred to as trueness.

Precision

Under defined set of conditions, the degree of agreement between values obtained from multiple measurements measured for a single homogenous sample is termed as precision. Repeatability, reproducibility and intermediate precision are the three levels of precision. The precision of an analytical procedure is usually expressed as the

variance, standard deviation (SD) or coefficient of variation (CV) of a series of measurements.

Linearity and Range

The ability of an analytical procedure to obtain results for the test substance that are in direct proportion to the analyte concentration is termed as linearity. When an analytical procedure has been demonstrated to be precise, accurate and linear, in that case the interval between the highest and lowest amount of analyte in test sample is termed as range.

Selectivity

The ability of an analytical method to measure only particular analyte when multiple analytes are to be detected in a given sample is termed as its selectivity.

Specificity

It is the capability of the method to estimate the analyte precisely in the presence of all possible sample or formulation components such as excipients, impurities, degradation products etc. (3) The specificity of an analytical method can be tested by estimating the analyte in a test sample followed by its comparison with standard solution that only contains an analyte. Any variation in the response of analyte in the test sample as compare to the standard solution suggests non-specificity of the analytical method.

Limit of Detection (LOD)

It is the lowest amount of sample that can be detected using the analytical procedure employed but not necessarily be quantitated.

The detection limit (DL) may be expressed as: $LOD=\frac{3.3\,\sigma}{S}$

Where, σ = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte.

Limit of Quantification (LOQ)

Under a suitable level of precision and accuracy, the lowest concentration of analyte in a sample that can be accurately quantified using the analytical procedure is termed as LOQ. It is of high importance for determination of impurity / degradation product in a sample.

The quantification limit (QL) may be expressed as: $LOQ=\frac{10\,\sigma}{S}$

A UV-Visible Spectrophotometric method was employed for determination of cisplatin loaded (as a complex) in HNCs of cisplatin and in-vitro drug release study. Cisplatin solution itself absorbs ultraviolet radiation at 203nm, 301 nm and 362 nm but values ofmolar extinction coefficients are 173, 4.33 and 0.806 respectively[i]. The low values of extinction coefficient indicate the very less sensitivity of the analytical method. Also the method would be hindered by the insolubility of cisplatin in commonly used solvents such as methanol and chloroform.

Cisplatin estimation in formulation was carried out using an established method of carrying out ligand exchange reaction between the cisplatin and the derivatizing reagent o-phenylenediamine(OPDA) and estimating the derivatized cisplatin at 705 nm (4). This ligand exchange reaction is very sensitive and is used for detection of cisplatin in UV method as well as HPLC method (5). Derivatized cisplatin absorbs UV-Visible radiation at two wavelengths 430 nm and 705 nm. The absorption maximum of 705 was used for estimating cisplatin due to significantly higher absorbace at this wavelength than at 430 nm. The method was validated for use by determining the interference, if any, of the formulation ingredients, derivatizing agent, or cisplatin itself (6).

3.2 MATERIALS AND INSTRUMENTS

3.2.1 Materials

Table 3-1 List of materials used with their sources

Materials	Source
Cisplatin	Sun Pharma Advanced Research Centre, Vadodara.
o-phenylenediamine(OPDA)	Merck, Germany.
Dimethylformamide(DMF)	S.D.Fine Chemicals, Mumbai
Hydrochloric acid	S.D.Fine Chemicals, Mumbai.
Acetic acid Glacial	S.D.Fine Chemicals, Mumbai.
Distilled Water	Prepared in Lab Distillation Assembly
UV-1600 spectrophotometer	Shimadzu, Japan.

3.2.2 Instruments

Table 3-2List of Equipment Used

Sr. No.	Equipment	Company
1.	Single pan electronic balance	Type AX 120 & ELB 300, Shimadzu

2.	UV Visible Spectrophotometer	UV-1800,Pharm Spec,Shimadzu,Japan
3.	HPLC	Agilent Technologies 1260 infinity II
4.	Bath sonicator	Sartorius, India
5.	Nanodrop	Thermofisher

3.3 METHODS

3.3.1 Estimation of Cisplatin using derivatization method by colorimetry

3.3.1.1 Solutions

- **Solvent for reaction product dilution:** Mixture of Dimethylformamide (DMF) and water (7:3, v/v) pH adjusted to 6.2 with hydrochloric acid solution (0.1 N HCl).
- **Cisplatin stock solution:** Cisplatin solution (100 µg/ml) was prepared by dissolving 10 mg of Cisplatin with 0.15 M HCL solution and making up to 100 mL with the same solvent. 10 mL of the resultant solution was transferred in another 100 mL volumetric flask and made up to 100 mL with the 0.15 M HCl solution to get Cisplatin stock solution of 10 µg/mL. This stock solution was taken for further dilutions to make calibration plot.
- **o-Phenylenediamine(OPDA) stock solution:** Stock solution of 21.64 mg/ml was prepared by dissolving 216.4 mg of OPDA in dimethylformamide(DMF) in a 10 mL volumetric flask and making up the final volume to 10 mL with DMF.

3.3.1.2 Obtaining Reaction Product between CDDP and OPDA and determining absorption maxima

0.5 mL CDDP stock solution (10 µg/mL in 0.15 M HCl) was mixed with 50 µL of OPDA stock solution (21.64 mg/ml in DMF) (molar concentration of OPDA 600 times that of CDDP). The mixture was heated for 30 minutes at 90°C, allowed to cool, and then final volume was made up to 10 mL with a DMF:Water mixture. The final concentration of CDDP in solution was 0.5 µg/ mL. The absorption spectrum was recorded against solvent system between the range of 300 nm-800 nm. The same method was used for preparation of reaction products at different levels of cisplatin for calibration plot generation.

3.3.1.3 Interference of Different Ingredients in Analysis:

The drug solutions (at highest level of cisplatin) that are to be estimated by the adopted derivatizing method were scanned between the wavelength range of 200nm-800nm. Interference of OPDA and lipids used in liposome formulation were checked by recording the spectra of following solutions (All the solutions were made up to final volume of 10 ml with DMF:Water mixture (7:3, v/v). Absorption curves of the following solutions are displayed below in Figure 1-6. All dilutions were made with DMF:Water solvent system except specified.

3.3.1.4 Procedure for calibration plot

The procedure described below was used for calibration plot formation. 0.5 mL distilled water, 0.5 mL, 1.0 mL, 1.5 mL, 2.0 mL, 2.5 mL, 3.0 mL and 3.5 mL of cisplatin stock solution were sampled into different 10 mL volumetric flasks. These solutions were treated with 50 µL, 50 µL, 100 µL, 150 µL, 200 µL, 250 µL, 300 µL and 350 µL of OPDA stock solutions respectively. Each volumetric flaks were capped with lid and heated at 90°C for 30 minutes in water-bath and then allowed to cool to room temperature. The solutions were then made up to 10 mL with DMF: Water mixture (7:3, v/v, pH 6.2). Absorbance values of each of the solutions were recorded at 705 nm using solvent (DMF: Water 7:3) as blank. The experiment was repeated for 3 times. The mean absorption values with standard deviation were calculated and mean values were plotted against the concentration of Cisplatin to get the calibration plot (7).

3.3.2 Estimation of Cisplatin using High Performance Liquid Chromatography (NP-HPLC)

High Performance Liquid Chromatography (HPLC) method was used for estimation of Cisplatin in biological samples as it is a sensitive method and it can detect low concentration of drug.

3.3.2.1 Calibration plot of cisplatin caprylate

Estimation of Cisplatin was performed by normal-phase HPLC method/NP-HPLC (Agilent Technologies 1260 infinity II) using L8 column (250 mm *4.6 mm* 5 µm, Thermo scientific) at ambient temperature. The mobile phase ethyl acetate, methanol, dimethylformamide, and degassed water (25:16:5:5) was run at a flow rate of 1.0 ml/min. Samples were prepared by appropriate dilutions of Cisplatin in

dimethylformamide and final dilution was done with mobile phase. 20 µl of each solution was injected into HPLC and chromatogram was run for record of chromatogram. Estimation of Cisplatin was done using UV-visible detector at wavelength of 310 nm.

- **Preparation of Stock Solution**

Dissolve about 10 mg of Cisplatin, accurately weighed, in dimethylformamide in a 10-mL volumetric flask, dilute with dimethylformamide upto 10 ml volume, and mix.

- **Preparation of Working Standard Solutions**

0.1 ml of above solution was taken in 10 ml volumetric flask and diluted upto the 10 ml with mobile phase to produce 10 µg/ml of working standard solution.

Table 3-3NP-HPLC Process Parameters

Parameter	Value
HPLC	Agilent Technologies 1260 infinity II
Column (L8)	250 mm *4.6 mm* 5 µm, Thermo scientific
Wavelength	310 nm
Flow-rate	1 ml/min.
Run-time	10 min
Injection volume	Rhenodyne 7725 injector valve with fixed loop at 20 µl
Mobile Phase	Ethyl acetate, methanol, dimethylformamide, and degassed water (25:16:5:5)
Retention time	6.2 min

3.3.2.2 Calibration curve of Cisplatin caprylate in Rat plasma by NP-HPLC method

- **Collection of blood and separation of plasma**

Blood was collected from retro-orbital plexus of Swiss albino rats weighing 200-250 gm. Clear supernatant plasma was separated from blood after centrifugation at 5000 rpm at 4℃ for 10 min using REMI cooling centrifuge. Samples were kept at -70℃ till further analysis.

- **Preparation of Stock Solution**

Dissolve about 100 mg of Cisplatin, accurately weighed, in dimethylformamide in a 100-mL volumetric flask, dilute with dimethylformamide to volume, and mix.

- **Preparation of Working Standard Solutions**

0.1ml of above solution was taken in 10 ml volumetric flask and diluted upto the 10 ml with mobile phase to produce 10 µg/ml of working standard solution.

- **Preparation of Plasma drug solution**

Plasma calibration standards were prepared by spiking 20 µl of above working standard solutions of Cisplatin in 380µl of drug free plasma to obtain different concentrations of Cisplatin. Blank sample was prepared by spiking 20 µl of mobile phase in 380 µl of plasma. All solutions were stored at 2-8℃ until further use.

- **Calibration curve of Cisplatin in rat plasma by liquid-liquid Extraction Technique**

A simple single step protein precipitation method was followed for the extraction of drug from plasma. To the above plasma drug solution 1200 µl ice cold DMF was added to obtain different concentrations. The samples were then vortexed for 2 min and centrifuged at 5000 rpm for 10 min. Supernatant was collected and filtered through 0.22 µm nylon syringe filter. From this 20 µl was injected in HPLC and analyzed using above mentioned parameters.

Table 3-4Process Parameters

Parameter	Value
HPLC	Agilent Technologies 1260 infinity II
Column (L8)	250 mm *4.6 mm* 5 µm, Thermo scientific
Wavelength	310 nm
Flow-rate	1 ml/min.
Run-time	10 min
Injection volume	Rhenodyne 7725 injector valve with fixed loop at 20 µl
Mobile Phase	Ethyl acetate, methanol, dimethylformamide, and degassed water (25:16:5:5)
Retention time	5.9 min

3.3.2.3 Calibration curve of Cisplatin in lung homogenate by HPLC method (Normal Phase Chromatography)

- **Lung Homogenate Preparation:**

2.5 gm of lung tissue was homogenized in 25 ml distilled water with the help of high speed homogenizer in order to produce 10% of lung homogenate using ice bath to maintain temperature at 4℃. Clear supernatant of the homogenate was separated after centrifugation at 5000 rpm at 4℃ for 10 min. Samples were kept at -70℃ till further

analysis. Extraction of drug and process parameters are same as procedure for rat plasma sample preparation described in section 3.3.2.2.

3.3.3 Estimation of total phospholipid content by Stewart method

Phospholipid content may be estimated by Stewart's Colorimetric Method (8). This method for the detection of phospholipids relies on the ability of phospholipids to form a stable complex with ammonium ferrothiocyanate. Ammonium ferrothiocyanate is a red coloured inorganic compound which has tendency to remain insoluble in chloroform. However, it can form stable complexes with phospholipids and those complexes are soluble in chloroform. Thus, when a chloroform solution consisting of phospholipids was mixed with ammonium ferrothiocyanate at room temperature, a red coloured complexes are formed which partitions in the chloroform phase and gets solubilized. The absorbance of the coloured soluble complex in chloroform can be determined at 462 nm wave length through colorimetry.

- **Preparation of ammonium ferrothiocyanate solution:**

27.03 g of ferric chloride hexahydrate ($FeCl_3.6H_2O$) and 30.4 g ammonium thiocyanate (NH_4SCN) was dissolved in deionised distilled water and volume was made up to 1 litre.

- **Calibration plot of Phospholipids:**

All the glass apparatus used for the study were washed with chromic acid solution prior to use to evade the probable contamination from surface active cleansing agents. A stock solution of 21.90 mg lipid mixture was prepared by solubilizing all the lipids in 21.90 ml of chloroform leading to final lipid concentration of 1000 µg/ml. The lipids used to prepare stock solution were those lipids used in the final optimized formulation with their respective molar ratios i.e. 105µl DPPC, 45 µl of Cholesterol, 15 µl of DOTAP, 15 µl of DOPE and 12 µl DSPE mPEG2000. The above lipid volume was taken from the different stock solution of lipids in chloroform having concentration of 100 mM. From this stock solution, 0.06, 0.12, 0.18, 0.24, 0.30 ml were taken which has 20, 40, 60, 80, 100µg/ml amount of lipids respectively and were added to 3 ml of ammonium ferrothiocyanate solution. Then chloroform was made up to the volume of 3 ml. The biphasic mixture was mixed thoroughly for 5 min and the high density lower chloroform layer was separated. The optical density of separated chloroform layer was measured at wave length of 462 nm by taking native chloroform

as a blank. The average of optical densities obtained was plotted in a graph against total lipid concentration.

3.3.4 Analytical method development of siRNA

3.3.4.1 UV spectrophotometric analysis of siRNA using nanodrop

The absorption spectroscopy is relatively insensitive and with conventional laboratory setup requires a nucleotide concentration of at least 1 ug/ml (or ng/µl). In order to fulfil this requirement, the traditional spectrophotometers cannot be used due to larger sample volumes, therefore, NanoDrop was used to estimate quantity and purity of siRNA samples in our experiments (9, 10). In addition, NanoDrop measures absorbance at 320 nm to detect any light-scattering components in the sample. The software subtracts the reading at 320 nm wavelength from the 260 nm, 280 nm and 230 nm values as background noise. After siRNA was confirmed for its purity then a correlation curve was constructed for method verification using this siRNA. siRNA stock solutions were prepared in nuclease free water (NFW) as per following: 2 pmol/µl, 4 pmol/µl, 6 pmol/µl, 8 pmol/µl and 10 pmol/µl. The main stock solution concentration was 10 µM and 1 µM solution was prepared by 10X dilution using NFW. 10 µM siRNA solutions equals 10 pmol/µl in equivalence with 133 ng/µl siRNA as per theoretical calculation. Aliquots of siRNA solutions were prepared accordingly to make 2-10 pmol/µl. The obtained nucleic acid concentration (siRNA) by nanodrop was plotted against concentration of siRNA. The general sample quantity requirement for experiments was 10 ul, which ensured minimum quantity of siRNA.

Preparation of Nuclease Free Water (NFW):

Diethylpyrocarbonate (DEPC) is commonly used reagent for preparation of NFW, buffer and other solutions. DEPC is an alkylating agent which reacts with -NH, -SH and -OH groups in RNases and other proteins, resulting in enzyme/nuclease inactivation (11). Therefore, all the reagents and solutions used in RNA work were prepared in DEPC treated/Nuclease free water. For this purpose, 0.1% of DEPC was mixed with 1 L doubled distilled water and stirred overnight at room temperature on magnetic stirrer. The dispersion was then autoclaved at 121 °C and 15 psi for 20 min to breakdown the residual DEPC and prepare NFW.

3.3.4.2 Gel electrophoresis: siRNA quantification by gel retardation assay

In order to maintain bio-activity of siRNA it is essential that the siRNA be delivered in its intact form. The siRNA is highly unstable biomolecule and the intact nature of siRNA can be lost due to hydrolysis. In contrast to DNA, the presence of 2'-OH groups promote RNA hydrolysis under acidic and basic conditions. This results in formation of smaller fragments of siRNA from the initial intact molecule (11). Therefore, it is vital that the prepared formulation, as well as the developmental process be carried out in presence of a method to detect the impact on the integrity of siRNA.

Electrophoresis is generally used to identify, quantify, and purify nucleic acid fragments and assess quality. The gel electrophoresis works on the principle that when charged molecules are placed in electric field, they travel towards oppositely charged electrode according to their charge. As nucleic acids have a considerable negative charge due to phosphate backbone, they migrate towards the anode in a matrix of "gel" (9). The gel run by immersing in an electrophoresis buffer containing ions to conduct current and a buffer to maintain the pH. For RNA applications the gel is composed of agarose at concentrations of 0.5 to 2% for DNA and mRNA analysis. The agarose is a seaweed polysaccharide obtained from Gelidium and Gracilaria, and consists of repeated agarobiose (L-and D-galactose) subunits . The gel can be easily prepared by melting the agarose and pouring it into the slab. During gelation, agarose chains interact non-covalently to form a network of pores which determine a gel's molecular sieving properties. The higher the agarose concentration the "stiffer" the gel. The agarose concentration has to be chosen to suit the particular application.

The detection of siRNA bands can be done by different techniques. The nucleic acid molecules can be visualized under UV light (>2500 μW/cm2) after staining with an appropriate dye. Ethidium bromide is the most common dye used for this application. The ethidium bromide has property to intercalate between the base pairs which results stabilization of the phenyl moiety and a 20 fold increase in fluorescence of orange color corresponding to wavelength ~ 605 nm after excitation with UV light (~305 nm) (12). Generally, 200 ng of RNA amount is required for visualizing with ethidium bromide. However, the sensitivity of the method can be

improved by using stronger fluorescing dyes such as SYBR® Gold and SYBR® Green II RNA gel stain (13). A UV transilluminator is typically used for this purpose. The light transmitted by fluorescing nucleotide can be detected after blocking the UV light using an orange filter (14, 15).

The length of RNA or number of nucleotide determines its rate of migration in the gel, the longer RNA molecules move slower than shorter fragments. The loss of integrity is accompanied by siRNA fragmentation and loss of molecular weight. This change can be detected through gel electrophoresis and it is compulsory tool for siRNA quality evaluation. This can also give information about quantity of siRNA. The intact total siRNA when run on polyacrylamide gel produce sharp bands. Partially degraded siRNA appears as smear due to low molecular weight and lack sharp bands. While a completely degraded siRNA forms a very much diffused faint band. If there are secondary structures, the siRNA may not migrate according to its true size resulting in formation of multiple bands for a single RNA corresponding to different structures. Therefore, inclusion of RNA size marker molecules or control helps in determination of size and interpretation of results and also ensures that the gel was run properly.

Protocol:

The required quantity of agarose (2 %) was dissolved in 100 ml of 1x TBE (Tris-Borate-EDTA) buffer, by heating with intermittent shaking to obtain a clear solution. Melted agarose cooled just above the pourable consistency. Meanwhile, gel tray for casting was prepared and tightly fastened at both the ends with tape to form a fluid-tight seal and comb was placed in the gel casting tray. To the cooled agarose (45-60°C) Ethidium bromide was (0.5 µg/mL) added before pouring. Ethidium bromide loaded gel was casted with suitable height to contain a well volume of 50 ul. Gel was allowed to set at 20°C for 30 min. After solidification, the comb was removed carefully, without damaging the wells, and tapes were taken off the edges to ensure conductive path in chamber. Gel was submerged in electrophoresis chamber (Genet Electrophoresis Power pack, Bangalore, India) with electrophoresis buffer (1x TBE buffer). Initially, minimum detectable quantity of siRNA on gel was determined by starting from the lowest concentrations: 10 pmole. Then the method for relative

quantification based on densitometry was developed and verified by analyzing increasing standard concentration of siRNA in the detectable range of 2, 4, 6, 8 and 10 *pmol*. The concentrations were determined relative to reference band of 10 *pmol (relative band density 1)*. Different concentration of siRNA solutions were mixed with suitable quantity of NFW and 2 ul of 6X gel loading buffer to make final siRNA amount in the range of 2-10 *pmol*. The method was validated for reproducibility by running 3 multiples and evaluating the RSD. The siRNA concentration of 2, 4, 6, 8 and 10 pmole was run on gel with gel loading buffer and run on 2 % agarose gel at 50 V. siRNA was detected with Ethidium bromide (0.5 µg/ml) and visualized on UV transilluminator GelDoc™ XR+ Imaging System (BioRad, USA). The band density at 10 pmole was taken as reference i.e. 1 and remaining bands were quantified relative to it. The Image lab (version 5.2.1) software was used for densitometry analysis. Further, the correlation curve between concentration and relative band density was prepared (16).

3.4 RESULTS AND DISCUSSION

3.4.1 Estimation of Cisplatin using derivatization method by colorimetry

Table 3-5Calibration Plot of Cisplatin

Cisplatin Concentration (µg/mL)	Mean Absorbance Values ± Standard Deviation (n=3)
0	0.000± 0.000
0.5	0.124 ± 0.003
1.0	0.263 ± 0.001
1.5	0.431 ± 0.002
2.0	0.573 ± 0.004
2.5	0.712 ± 0.007
3.0	0.823 ± 0.004
3.5	0.951 ± 0.005

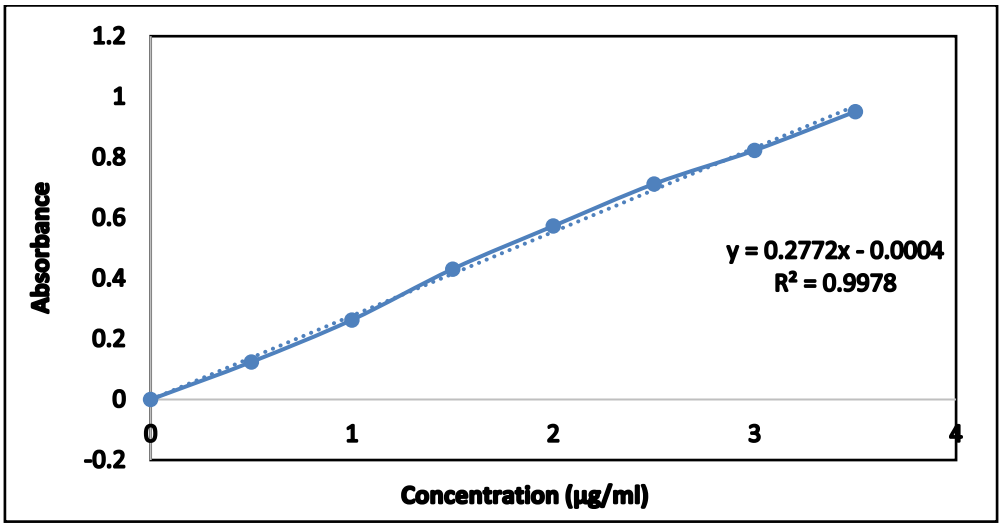

Figure 3-1Calibration Plot of Cisplatin derivatized with OPDA (in DMF: Water Mixture 7:3 v/v, pH 6.2)

As shown in the Figure 3-1, the calibration plot of Cisplatin in DMF:Water (7:3 % v/v) mixture was found to be linear in the concentration range from 0.5-3.5 ppm with high value of regression coefficient (R^2 = 0.9978) which shows that cisplatin obeys Beer's law between concentration range 0.5-3.5 ppm after derivatization with OPDA.

3.4.1.1 Accuracy

Table 3-6Accuracy of method with OPDA

(in DMF: Water Mixture 7:3 v/v, pH 6.2)

Level	Expected concentration (ppm)	Observed Concentration (ppm) ±SD (n=3)	% Drug recovered
80%	1.6	1.57±0.0032	98.12%
100%	2	2.04±0.0041	102%
120%	2.4	2.43±0.0033	101.25%

The % recoveries for lower, intermediate, and higher concentration are given in table Table 3-6 for DMF: Water Mixture 7:3 v/v, pH 6.2. Their result showed that any minor change in the drug concentration in the solution could be accurately determined and estimated by the proposed analytical method.

3.4.1.2 Precision

Table 3-7Intraday and Interday precision of Cisplatin withOPDA (in DMF: Water Mixture 7:3 v/v, pH 6.2)

Concentration (ppm)	Observed Concentration (ppm) ±SD (n=3)		%RSD	
	Intraday	Interday	Intraday	Interday
1	1.01±0.0033	1.04±0.0021	0.3267%	0.2079%
2	2.10±0.0036	2.13±0.0024	0.1714%	0.1142%
3	2.96±0.0075	3.02±0.0048	0.2533%	0.1621%

The precision study was performed under the same operating condition for intraday and interday. In precision study, %RSD values obtained were less than 2.0% which suggests that these methods have good precision and reproducibility. From the table Table 3-7, % RSD indicated that the method is precise and there is no intraday and interday variability in the method.

3.4.1.3 Limit of detection & Limit of quantification

Table 3-8LOD and LOQ of Cisplatin with OPDA

(in DMF: Water Mixture 7:3 v/v, pH 6.2)

LOD [ppm]	LOQ [ppm]
0.0402	0.1339

3.4.2 Estimation of Cisplatin using High Performance Liquid Chromatography (NP-HPLC)

3.4.2.1 Calibration curve of Cisplatin in ethyl acetate, methanol, dimethylformamide, and degassed water (25:16:5:5) by NP-HPLC

Calibration plot for drug was obtained in range of 4-12µg/ml. Regression equation for standard curve was found to y = 11024x - 10383. Correlation Coefficient for the method was noted to be 0.9970 signifying existence of linear relationship between peak area and concentration of the drug. Retention time was 6.2 min.

Table 3-9Calibration curve of Cisplatin by NP-HPLC

Concentration (µg/ml)	Area (µV*s)±SD (n=3)	% RSD
4	35787 ±99.61	0.28
6	54395±128.03	0.24
8	76419± 153.41	0.20
10	98413± 258.53	0.26
12	124014 ± 150.04	0.12

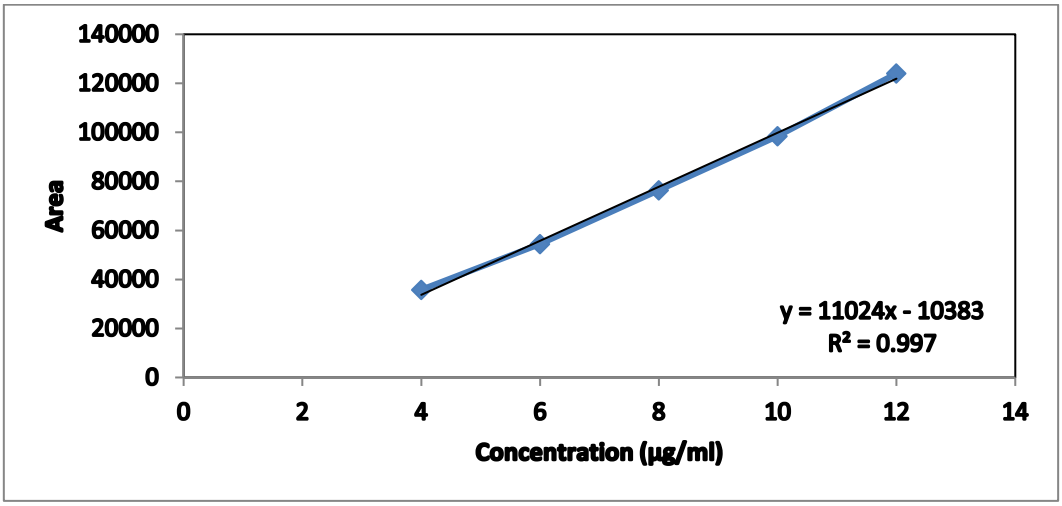

Fig. 3-2Calibration plot of Cisplatin by NP-HPLC

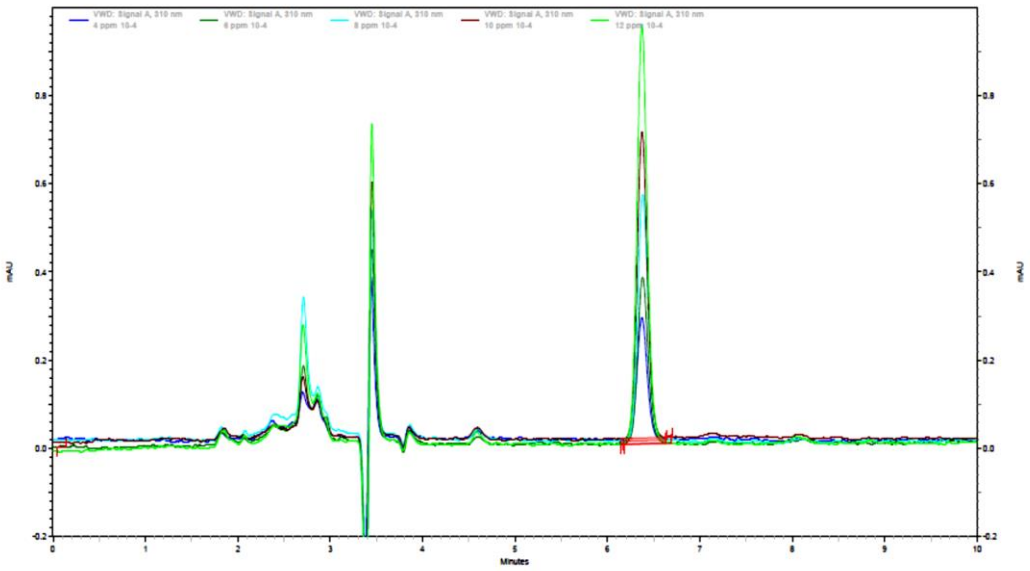

Figure 3-3 Overlay Chromatogram of Cisplatin by NP-HPLC

3.4.2.2 Accuracy

Table 3-9a Accuracy of RP-HPLC method

Accuracy Calculation							
Sr. No.	**Levels**	**Area**	**Amount Added**	**Amount Found**	**Amount recovered**	**Recovery**	**Mean recovery**
			(% w/w)	**(% w/w)**	**(% w/w)**	**(%)**	**(%)**
1	Control sample	3.6	0.0	-	-	-	-
2	Level-(150%) Sample Prep.-1	3861.2	499.0	454.5	453.0	90.8	**90.8**
3	Level-(150%) Sample Prep.-2	3873.9	499.0	447.0	446.5	89.5	**89.5**
4	Level-(150%) Sample Prep.-3	3891.2	499.0	443.0	442.8	88.7	**88.7**
5	Level-(50%) Sample Prep.-1	1322.3	166.3	143.0	142.0	85.4	**85.4**
6	Level-(50%) Sample Prep.-2	1316.2	166.3	145.0	144.5	86.9	**86.9**
7	Level-(50%) Sample Prep.-3	1320.2	166.3	140.0	139.5	83.9	**83.9**
8	Level-(20%) Sample Prep.-1	513.1	66.5	58.0	57.5	86.5	**86.5**
9	Level-(20%) Sample Prep.-2	509.6	66.5	59.0	60.5	91.0	**91.0**
10	Level-(20%) Sample Prep.-3	513.0	66.5	56.0	57.2	86.0	**86.0**
				Overall Mean		**87.6**	
				Overall SD		2.5	
				Overall RSD(%)		**2.8**	

Table 9-3b Linearity, LOD and LOQ of the developed method

Linearity, LOD and LOQ calculations		
% Level	Conc. (µg/ml)	Area
20	6.7	481.2
30	10.0	706.5
50	16.6	1228.4
80	26.6	1997.5
100	33.3	2393.1
120	39.9	2945.7
150	49.9	3625.3
Slop		73.06
Intercept		1.23
STEYX		35
CC		0.999
LOD		**1.58 µg/ml**
LOQ		**4.79 µg/ml**
(Experiments were done in triplicates)		

Table 9-3c Precision of method

Precision Calculation						
Sr. No.	**Levels**	**Area**	**Amount Added**	**Amount Found**	**Amount recovered**	**Recovery**
			(ppm)	**(% w/w)**	**(% w/w)**	**(%)**
1	Control sample	3.6	0.0	0.5	-0.5	0.0
2	Precision Set-1	2202.6	333.0	305.5	305.0	91.6
3	Precision Set-2	2187.7	333.0	303.7	303.2	91.0
4	Precision Set-3	2111.7	333.0	293.6	293.1	88.0
5	Precision Set-4	2094.0	333.0	292.2	291.7	87.6
6	Precision Set-5	2117.6	333.0	295.4	294.9	88.6
7	Precision Set-6	2179.0	333.0	301.9	301.4	90.5
			Overall Mean			**89.6**
			Overall SD			1.7
			Overall RSD(%)			**1.9**

3.4.2.3 Calibration curve of Cisplatin in Rat plasma by NP-HPLC method

Calibration plot for drug was obtained in range of 4-12µg/ml. Regression equation for standard curve was found to y = 8236.6x - 13545. Correlation Coefficient for the method was noted to be 0.9935 signifying existence of linear relationship between peak area and concentration of the drug. Retention time was 6.9 min.

Table 3-10 Calibration curve of Cisplatin in rat plasmaby NP-HPLC

Concentration (µg/ml)	**Area (µV*s)±SD (n=3)**	**% RSD**
4	21891 ± 163.79	0.75
6	34093 ± 195.34	0.57
8	50483 ± 241.21	0.48
10	67936± 449.01	0.66
12	87336 ± 366.07	0.42

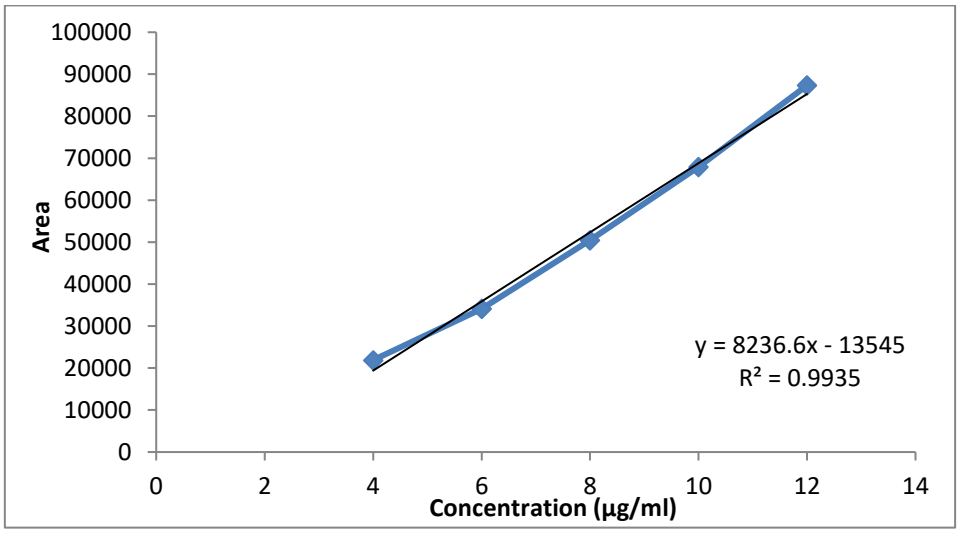

Figure 3-4Calibration plot of Cisplatin in Rat plasma by NP-HPLC

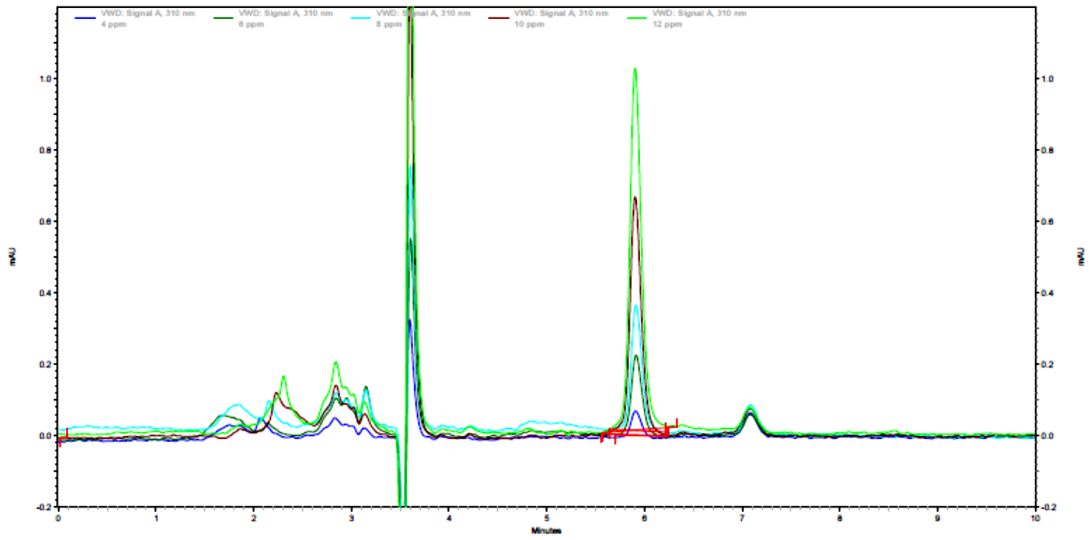

Figure 3-5 Overlay Chromatogram of Cisplatin in Rat plasma by NP-HPLC

3.4.2.4 Calibration curve of Cisplatin in Lung homogenate by NP-HPLC method

Calibration plot for drug was obtained in range of 4-12 µg/ml. Regression equation for standard curve was found to y = 6686.9x - 8784.3. Correlation Coefficient for the method was noted to be 0.9943 signifying existence of linear relationship between peak area and concentration of the drug. Retention time was min.

Table 3-11Calibration curve of Cisplatin in Lung homogenate by NP-HPLC

Concentration (µg/ml)	Area (µV*s)±SD (n=3)	% RSD

0	00000 ± 0000	000
4	19754 ± 233.59	1.18
6	30171± 212.96	0.71
8	43380± 376.45	0.87
10	57079± 629.66	1.10
12	73169 ± 669.98	0.92

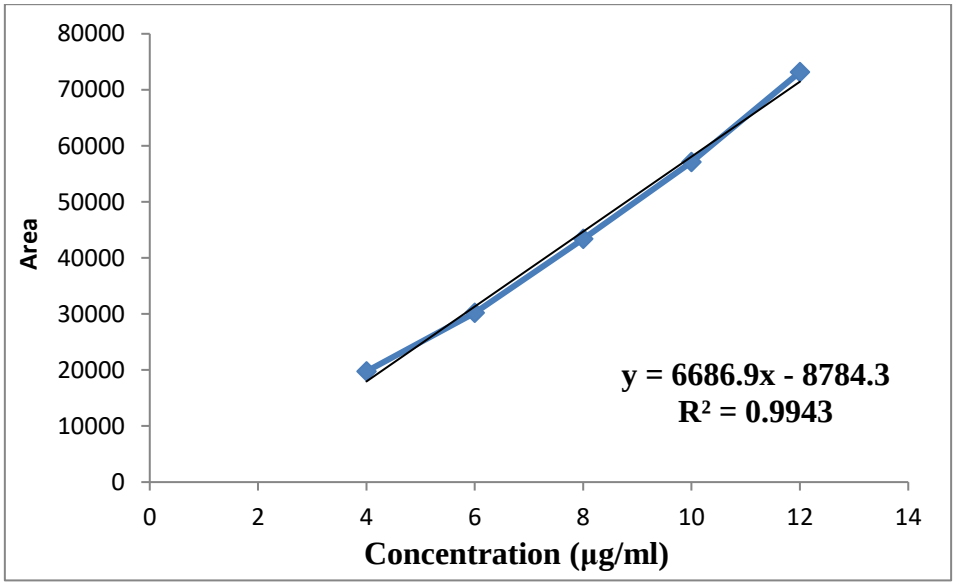

Figure 3-6Calibration plot of Cisplatin in Lung homogenate by NP-HPLC

3.4.3 Estimation of total phospholipid content by Stewart method

This method was used to quantify the total amount of lipids in formulation. All the lipids that were used in final composition for preparation of HNCs were dissolved in chloroform to prepared stock solution. Dilutions of the stocks were prepared and colorimetric estimation of ammonium-ferrothiocyanate: lipid complex was performed to obtain calibration curve.

Table 3-12Calibration data for estimation of total phospholipid content

Concentration (ppm)	Absorbance ±SD (n=3)	% RSD
0	0.000± 0.0000	0.000 %
20	0.227± 0.0018	0.609 %
40	0.420± 0.0011	0.342 %
60	0.611±0.0045	1.290 %
80	0.815± 0.0076	0.310 %

100	0.993± 0.0014	0.180 %

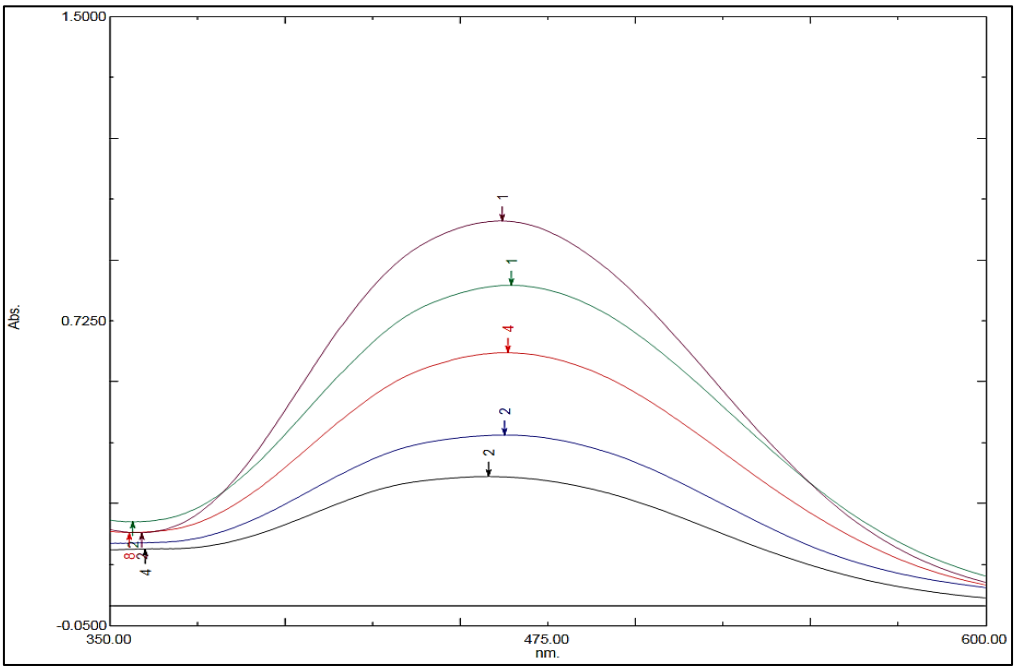

Figure 3-7Calibration plot of total phospholipids mixture in chloroform

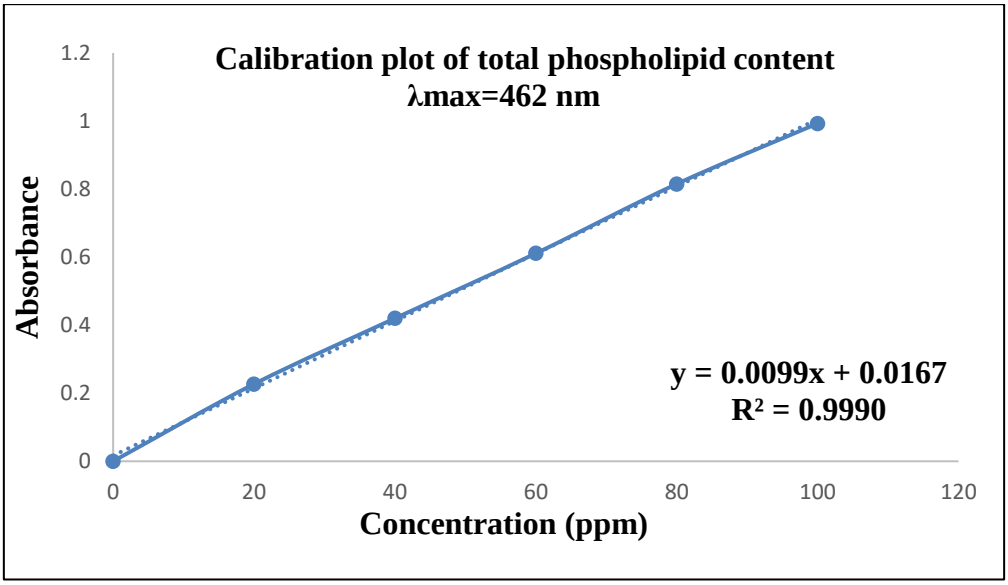

Figure 3-8 Calibration plot of total phospholipids mixture in chloroform

As shown in the Table 3-9, the calibration plot of total phospholipids mixture in chloroform was found to be linear in the concentration range from 20-100 ppm with high value of regression coefficient (R2 = 0.9990) which shows that the phospholipids obeys Beer's law between concentration range 20-100 ppm.

3.4.4 Analytical method development of siRNA

The siRNA meets the purity requirements as the A260/A280 and A260/A230 are within the acceptable range which is depicted in chapter 4 section. The correlation between prepared and obtained concentration showed that the method can be used for determination of concentrations from minimum of 0.5 to avoid instrument noise up to 8 pmole/ul to avoid detector saturation as the absorbance was in the range of 0.2 to 1.2. This was linearity range for analysis of siRNA by using Nanodrop (Table 3-13).

3.4.4.1 UV spectrophotometric analysis of siRNA

Table 3-13 Obtained concentration of siRNA at 260 nm

Concentration of siRNA (pmole/µL)	Obtained Concentration by Nanodrop (ng/ µL) ±SD (n=3)
2	25.28±0.548
4	50.83±1.271
6	73.95±0.321
8	95.28±0.193
10	127.64±1.294

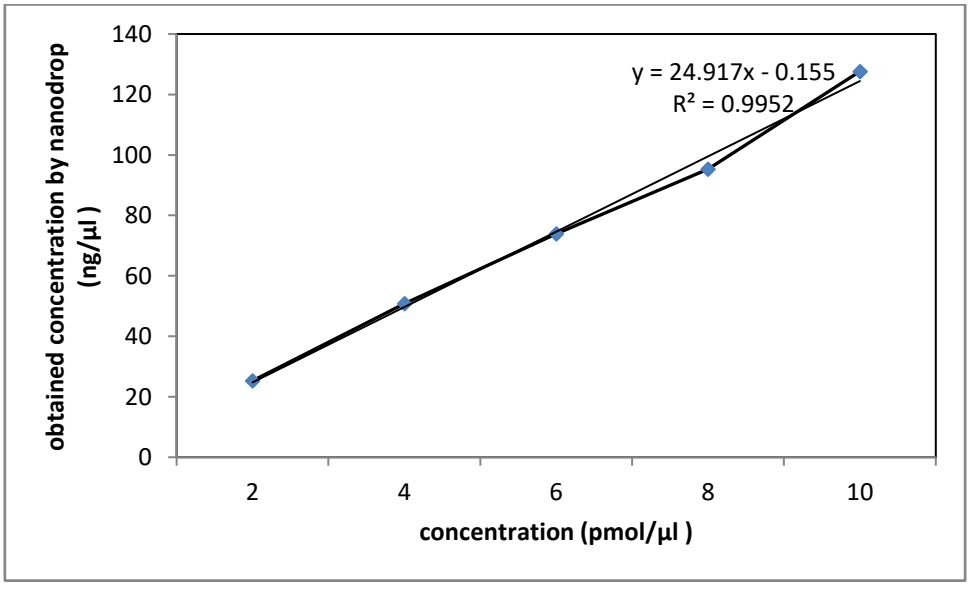

Figure 3-9Correlation of known concentration of siRNA (pmol/µl) Vs. obtained concentration (ng/µl) by Nanodrop results

3.4.3.1 Gel electrophoresis: siRNA quantification by gel retardation assay

The gel electrophoresis was proposed to study the integrity of siRNA and the complexation capacity of vectors. As the vectors being studied had pH dependent ionization, the electrophoresis buffer pH was decided to be 7.4 as it is physiologically relevant condition. The target was to get sharp bands for pure siRNA. In the first step the agarose concentration was optimized and it was observed that the lower agarose concentrations result in band distortion. When the agarose concentration was increased to 3 %, we obtained condensed, crisp band for siRNA. Therefore, the agarose concentration of 2 % was finalized for all the experiments.

The pH adjustment of TBE buffer was also important step. The pH of unadjusted 1X TBE buffer is around 8.0. Therefore, it has to be acidified with suitable acid to decrease the pH to 7.4. The pH adjustment with Hydrochloric acid was the direct option. However, it was observed that pH adjusting agent such as HCl, containing ions of higher conductivity, and result in significant increase in conductivity. This increase in conductivity drags the siRNA in its elution path and results in band distortion. Therefore, pH adjusting agent with lower conductivity was proposed for pH adjustment such as Acetic acid. The gel run in electrophoresis buffer containing acetic acid as pH adjusting agent did not result in band distortion which was attributed to the lower conductivity of acetate anions (0.0409 S L mol-1 cm-1) compared to chloride anion (0.07635 S L mol-1 cm-1). The change in conductivity was apparent from the readings of Ampere on the current supplier at constant voltage of 50 mV. All other parameters were set as per following according to optimized procedure given by Patil et al [14].

Table 3-14Optimized parameters for gel electrophoresis

Parameter	Optimized condition	Comments
Detectable siRNA	1 pmole	Lower are unsuitable for quantification
Tank buffer	TBE, 7.4 pH	pH adjusted with Acetic acid for low current
Voltage, Current	50 V, 35 mA	Higher voltage caused dragging effect
Vehicles for siRNA	0.05% sodium acetate	Higher conc. resulted in band distortion
Agarose Gel	2 % w/w	Lower conc. resulted in drag effect

Run time	40 min	Required for 3/4th run

- **Quantification:**

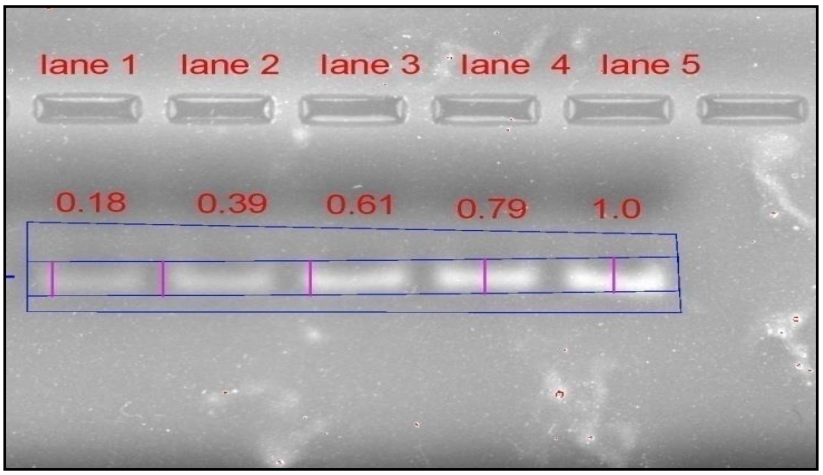

Figure 3-10Gel electrophoresis band density at different siRNA concentration

(Lane 1: 2 pmol, lane 2: 4 pmol, lane 3: 6 pmol, lane 4 8 pmol, lane 5: 10 pmol)

Table 3-15Relative band densities at different siRNA concentrations

Concentration of siRNA(pmole)	Relative band density±SD (n=3)
2	0.18±0.238
4	0.39±1.194
6	0.61±0.927
8	0.79±1.983
10	1.0
*Values are represented as mean±SD, n=3	

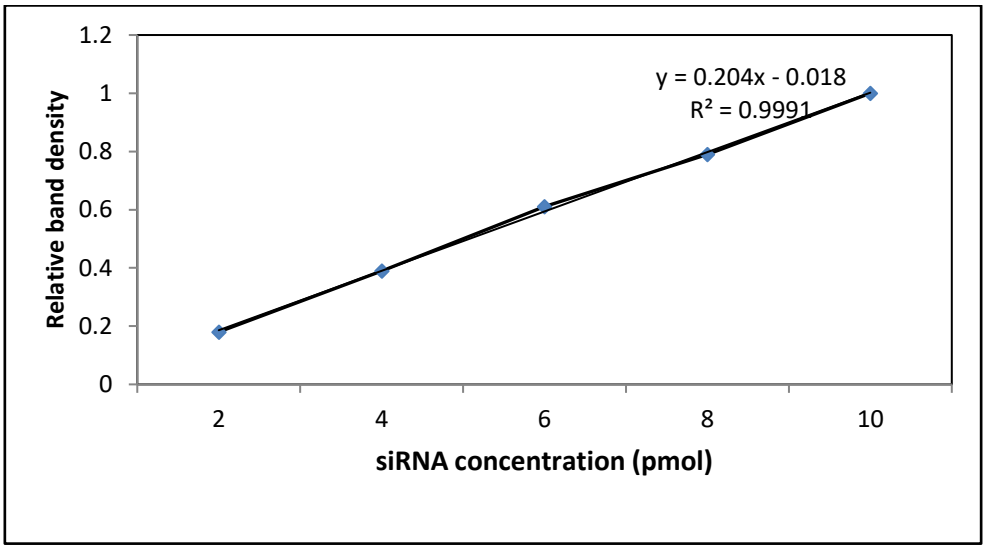

Figure 3-11 Calibration curve for prepared siRNA concentration and relative band density

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Table 3-15 showed correlation between concentration and obtained band density. The RSD values for all the densitometry analysis were < 2.0%. Further, the correlation curve between concentration and relative band density was prepared (Figure 3-11 Calibration curve for prepared siRNA concentration and relative band density). It was observed that there was a perfect correlation with R^2 value of 0.993. Therefore, the method is accurate for estimations with use of internal standard in analysis.

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