4 PREFORMULATION STUDIES

4.1 INTRODUCTION

Preformulation experiments are intended to classify both the physiochemical properties of the medication and the excipients that can influence the production process, the nature of the formulation, and the pharmacokinetic properties of the resulting product. This may provide valuable knowledge or support the need for molecular alteration in the formulation design. Each medicine has inherent chemical and physical properties that are taken into consideration before the pharmaceutical formulation produced. This property establishes a basis for the manufacturing of medicines combined with medicinal ingredients in the form of dosages. The aim of the pre-formulation analysis is to formulate the elegant, stable, efficient and safe form of dosage by determining the kinetic rate profile, compatible with the other ingredients and the physico-chemical parameters of new drug substances. Preformulation studies on shRNA pDNA has been used to determine working condition management during formulation.

4.2 LIST OF MATERIALS AND EQUIPMENTS

4.2.1 Materials

Table 4-1 List of materials

Sr no.	Name	Supplier of Material
1	Docetaxel	Gift sample from Sun Pharmaceuticals Advanced Research Centre (SPARC)
2	PEG-PCL	Gift Sample from PURAC England
3	1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC)	Gift samples from Lipoid, Germany
4	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy(polyethylene glycol)-2000] (sodium salt) (DSPE-PEG)	
5	Di-oleoyl trimethylammoniumpropane (DOTAP)	
6	Acetonitrile (Analytical Grade)	S D Fine Chemicals, India

Table 4-2 Equipments and Instruments list

Sr. No.	Equipment	Manufacturer
1.	Digital Weighing Balance	Shimadzu, Japan
2.	Melting point apparatus	VEEGO, Mumbai
3.	UV visible Spectrophotometer	UV-1700 Shimadzu, Japan

4.	Motorized pellet press	Kimaya engineers, India
5.	FT-IR spectrophotometer	Bruker, Japan
6.	Differential scanning calorimeter (DSC 60)	Shimadzu, Japan
7.	Gel electrophoresis unit	Bio-Rad, USA.
8.	Gel imaging chamber	Bio-Rad, USA.

4.3 CHARACTERIZATION OF DOCETAXEL

4.3.1 Organoleptic characterization

Visually, docetaxel drug organoleptic characterization was performed and examined for unique characteristics such as shape, structure, color and odor.

4.3.2 Melting Point Determination

4.3.2.1 Melting point determination by Glass Capillary Method

Confirmation of the melting point is the key criterion for verified drug sample purity. By applying a small volume of docetaxel to a capillary tube enclosed at one end, the melting point was determined. The capillary tube was kept in an electrically controlled melting point apparatus (VEEGO, Mumbai) in which the temperature at which Docetaxel melts was recorded. Docetaxel identity was verified by comparison of the obtained value with the recorded melting point value for the Docetaxel (1).

4.3.2.2 Melting Point Determination by Differential Scanning Calorimetry (DSC)

Differential Scanning Calorimetry (DSC) also determined the melting point of the substance (DSC). Docetaxel thermography was obtained using DSC (DSC-60, Shimadzu, Japan). (DSC-60, Shimadzu, Japan). The compound was enclosed in an aluminium pan and heated under nitrogen purification at a steady rate of 10 °C/min over a temperature range of 30-300 °C with nitrogen flow rate of 30 ml/min to maintain an inert atmosphere and to prevent oxidation (2).

4.3.3 Infrared Spectroscopy

By preparing a Potassium Bromide (KBr) pellet, the IR-spectrum of Docetaxel was measured in the solid state. Pure docetaxel was grounded and extensively mixed with a 1:100 (sample KBr) ratio of KBr, an infrared transparent matrix. KBr pellets were prepared by adding a pressure of 10-12 metric tons to the motorized pellet press (Kimaya engineers, India). The

pellets were then scanned over a 4000-400 cm-1 wavelength range and the spectrum was collected using the FTIR spectrometer-430 (Bruker) (3).

4.4 DOCETAXEL-EXCIPIENTS COMPATIBILITY STUDIES

4.4.1 DSC

A Differential Scanning Calorimeter (DSC-60, Shimadzu, Japan) with a heating rate of 20 °C per minute in the 25-300 °C range under the inert nitrogen atmosphere at a flow rate of 40 ml/min was used for the DSC study. DSC thermograms for Docetaxel, PEG-PLA, Lipid mixture (DPPC: DOTAP: DSPE-PEG_{2000}) and physical mixture (Docetaxel: PEG-PLA : lipid mixture at a 1:1 ratio) were performed to verify drug interaction with excipients.

4.4.2 IR

The 1:1 ratio of Docetaxel: PEG-PLA : lipid mixture (DPPC: DOTAP: DSPE-PEG_{2000}) was prepared and scanned using FT-IR spectrometer-430 for the compatibility analysis of Docetaxel and excipients (Bruker).

4.5 shRNA pDNA WORKING CONDITION OPTIMIZATION

Stability of working stock solution (50ng/0.02ml) of shRNA pDNA was assessed after repeated sampling along with presence/absence of DNase/RNase. Samples were taken from the manufacturer's sample vial and checked for the integrity of shRNA pDNA after repeated sampling. Moreover, samples were withdrawn from working stock and DNase/RNase were added to the solution and evaluated for shRNA pDNA integrity using gel electrophoresis. Stability of shRNA pDNA was also assessed to evaluate effect of working condition's temperature and pH. To understand effect of pH and temperature, 50 ng/0.02 ml stock solution of the shRNA pDNA were kept at different temperature ranging from 30°C to 70°C. shRNA pDNA solution has been tested under weakly alkaline condition at pH 7.5 TRIS buffer and weakly acidic condition with pH 6.8 TRIS buffer (4).

4.6 RESULTS AND DISCUSSION

4.6.1 Organoleptic characterization of Docetaxel

Table 4-3 Estimation of organoleptic characteristics

Sr. No.	Parameters	Results	Inference
1.	Color	White	Matched with Docetaxel standard
2.	Odour	Odorless	Matched with Docetaxel standard
3.	State	Crystalline powder	Matched with Docetaxel standard

4.6.2 Melting point determination

4.6.2.1 Glass Capillary Method

The docetaxel melting point evaluated by the Glass capillary method was found to be in the 176 °-182 ° C range, which was matching with the 176-190 ° C range of recorded value.

4.6.2.2 Differential Scanning Calorimetry (DSC)

The Docetaxel DSC thermogram in Figure 4-1 shows a high endothermic spike at 179 °C equivalent to the its melting point. The melting point graph found using the capillary process is also verified by this. (176-182 ° C) (5)

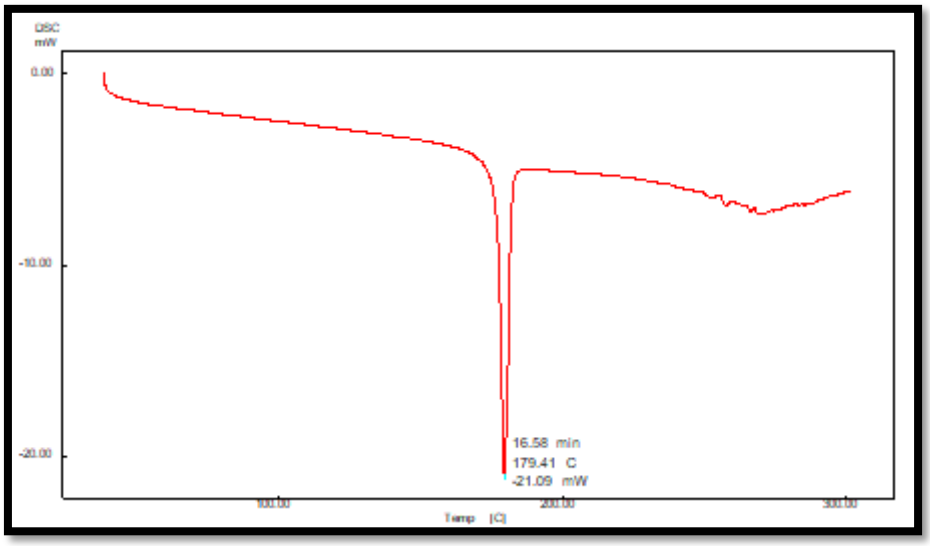

Figure 4-1 DSC Thermogram of Docetaxel

Table 4-4 Observation of Docetaxel Melting Point by different methods

Sr. no.	Method	Observed Melting Point
1.	Glass Capillary method	176°-182°C
2.	Differential Scanning Calorimetry	179 °C

4.6.3 Authentication of drug by FTIR

The practically obtained Docetaxel IR spectra was compared with the standard Docetaxel IR spectra (Figure 4-2), which was observed to have all the characteristic peaks of the functional groups. So, Docetaxel was confirmed to be authentic.

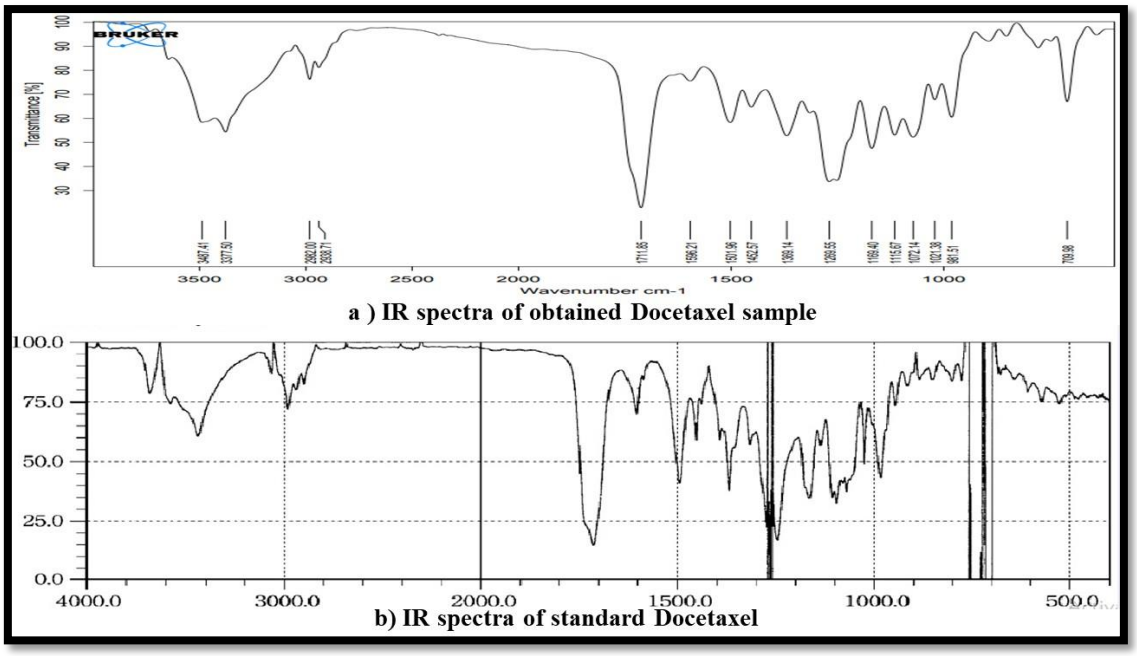

Figure 4-2 a) IR spectra of Docetaxel sample Vs. b) IR spectra of standard Docetaxel

Table 4-5 IR spectrum data of Docetaxel

Observed frequency(cm-1)	Reported frequency(cm-1)	Interpretation of Functional Group
3377.50	3550-3200	OH stretching
3487.00	3350-3310	NH stretching
1452.57	1650-1350	C=C stretching
1711.85	1720-1760	C=O Stretching
2982.00	2840-3000	C-H stretching

4.6.4 Drug-excipients Compatibility Study

4.6.4.1 Compatibility study by DSC

In thermal analysis, Differential Scanning Calorimetry (DSC) is generally used to track endothermic (melting, solid-solid phase transformations and chemical degradation) and exothermic processes (Crystallization and oxidative decomposition) (6). In pre-formulation tests, it is highly useful as it suggests the presence of potential association between drug-excipient or excipient-excipient in formulation.

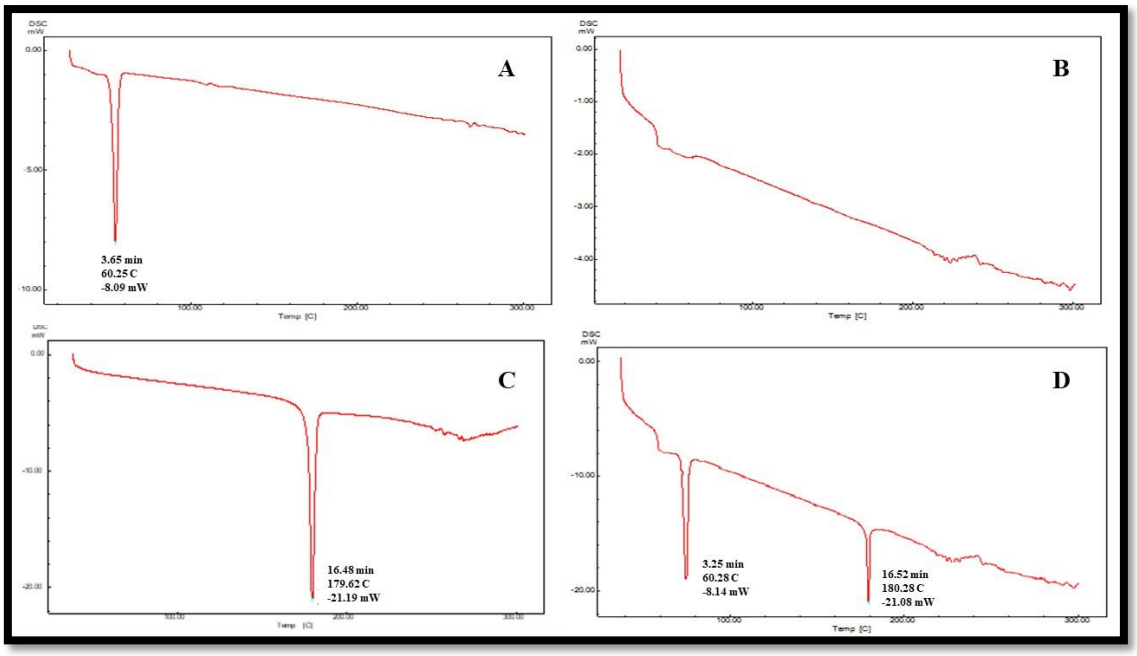

Figure 4-3 DSC thermogram for Compatibility study

(A) DSC thermogram of PEG-PCL (B) DSC thermogram Lipid admixture (DPPC: DSPE-PEG2000: DOTAP) (C) DSC thermogram of Docetaxel (D) DSC thermogram of physical mixture of Docetaxel and excipients)

DSC Docetaxel thermogram displayed a high endothermic peak corresponding to Docetaxel melting point at 179.62 °C. Whereas a sharp endothermic peak at 60.25 °C shows the melting point equivalent of PEG: PCL diblock co-polymer. However, DSC thermogram of the DPPC: DSPE-PEG_{2000}: DOTAP (lipid admixture) doesn't show any peak due to negative glass transition temperature of resultant lipid mixture. DSC thermogram of physical mixture of Docetaxel, PEG-PCL and lipid admixture demonstrates no change in the endothermic peak of Docetaxel. Hence it can be said that Docetaxel and excipients used in the formulation development are compatible with one another.

4.6.4.2 Compatibility study by Infrared spectroscopy

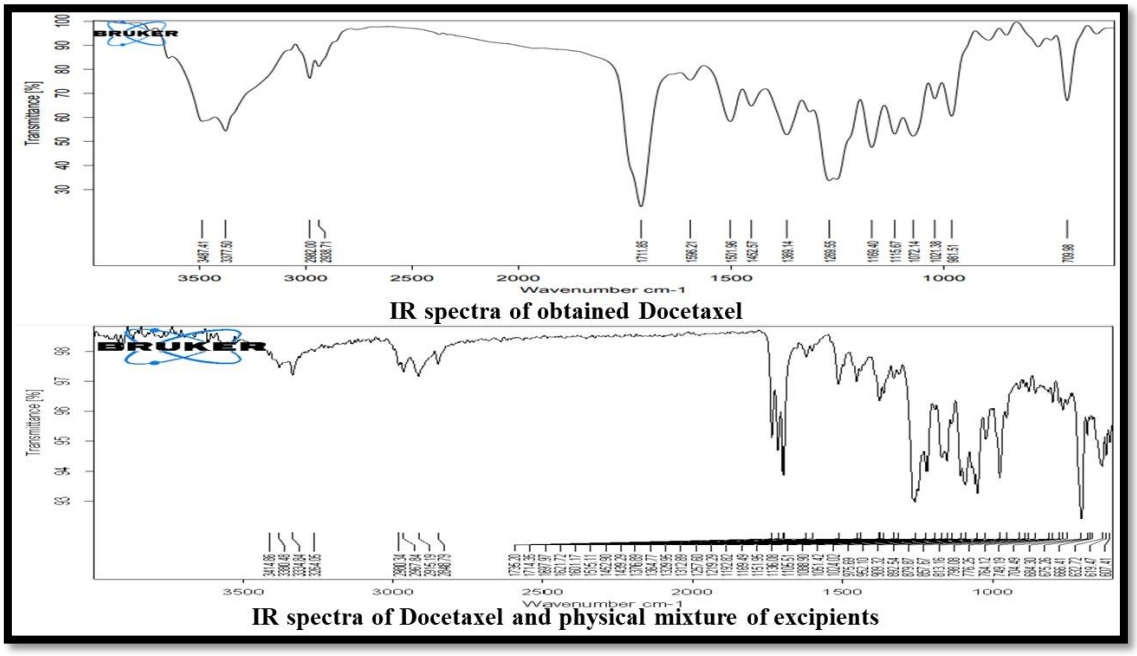

Figure 4-4 Compatibility study by infrared spectroscopy

The IR Spectrum of Docetaxel is shown and characteristic bands were identified in Figure 4-4. For the compatibility of drug and excipients, IR spectra of Docetaxel and physical mixture (Docetaxel +PEG-PCL+ DPPC: DSPE-PEG_{2000}: DOTAP) was studied as shown in Figure 4-4. IR graphs exhibited characteristic peaks at 3377.50 cm^{-1} for OH stretching, 3487.00 cm^{-1} for NH stretching, 1452.57 cm^{-1} for C=C stretching, 1711.85 cm^{-1} for C=O stretching, and 2982.00 cm^{-1} for CH stretching vibrations. All these peaks are considered characteristic to Docetaxel and are prominently observed in IR spectra of physical mixture as well. No additional peak was observed in physical mixture of drug and excipients. From these results, it was confirmed that there was no interaction between Docetaxel and physical mixture of excipients.

4.6.5 Optimization of working conditions for shRNA

Since shRNA plasmid is extremely unstable, the stability of the shRNA pDNA working solution was measured after three-repeated sampling from manufacturer's sample as shown in lane 1-3. DNase contamination during sampling may degrade the residual stock, which can result in degradation of shRNA pDNA. Consequently, the stability of the sampled secondary stock against the fresh primary stock sample was tested.

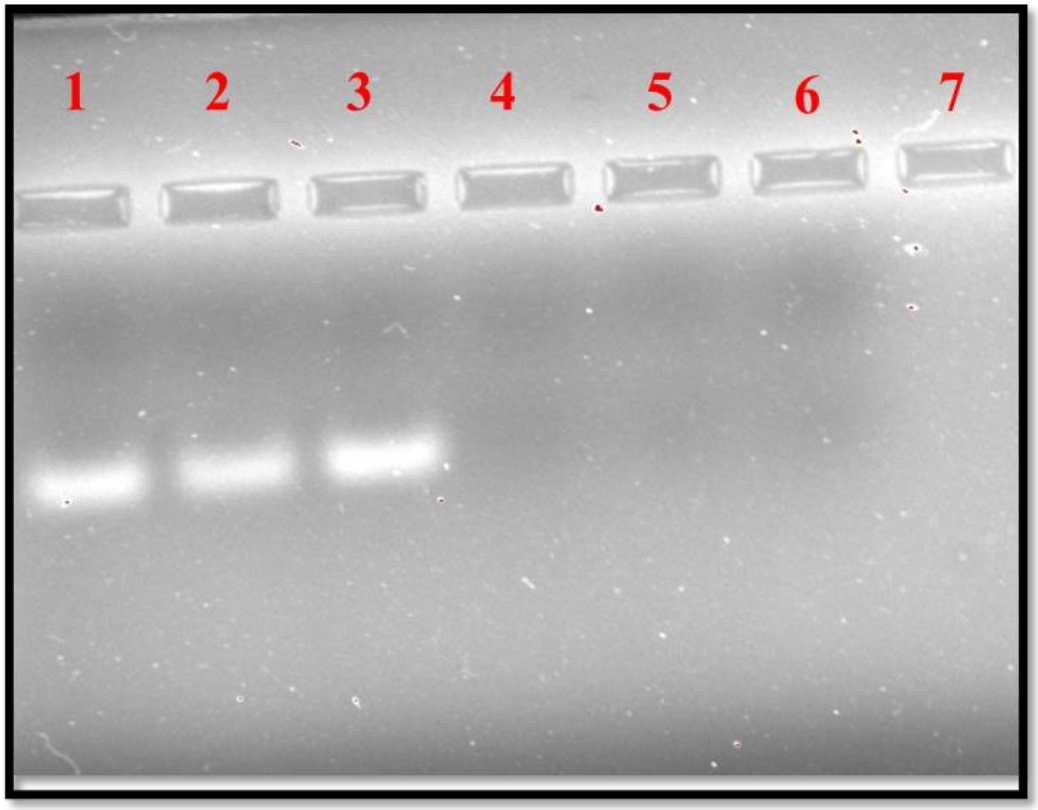

Figure 4-5 Stability of shRNA pDNA stock after repetitive sampling

In Figure 4-5, lane 1-3 suggests that the shRNA was unaffected and remained stable after repeated sampling from manufacturer's sample. Lane 4 to 7 in Figure 4-5 shows no band of shRNA pDNA plasmid which suggest degradation of pDNA. It was concluded that DNase and RNase added during sample preparation completely degraded shRNA pDNA shown in lane 4-5 and 6-7 respectively. Thus, shRNA pDNA molecules must get DNase and RNase free treatment. Hence, DEPC treatment was used to remove RNase and DNase from all materials before used for shRNA handling.

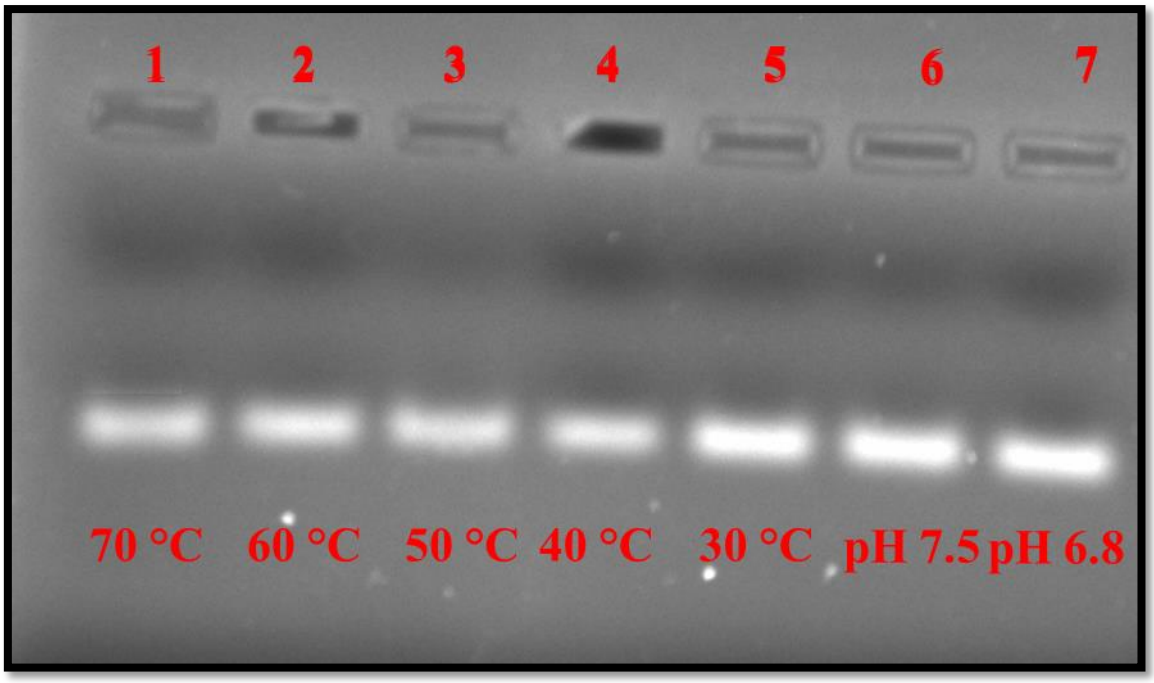

Figure 4-6 Effect of temperature and pH on shRNA pDNA

shRNA pDNA is prone to degradation with different heating condition and pH condition during formulation optimization. shRNA pDNA solutions were incubated for 30 min at different temperature. Lane 1-5 in Figure 4-6 shows that up to 20 % of shRNA were degraded over increase in temperature for 30 to 70 °C hence temperature above 70 °C must not be used during the any formulation optimization process. Lane 6 and 7 showed that shRNA pDNA has been stable over pH range of 6.8 to 7.5. The obtained range of pH and temperature conditions were found similar to manufacturer's conditions.

4.7 REFERENCES

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