4.PRELIMINARY STUDIES

4.1 INTRODUCTION

Preliminary studies were carried out to support and define variables limits and specifications related to HNCs. siRNA purity confirmation is essential for further formulation batches. DSC and FTIR studies are important in characterizing any interaction between drugs or drugs and excipients. This will give important information on whether the interaction, if sought, has taken place or not and also what would be the possible interaction. This would be helpful in assuring whether the optimal drug delivery has been developed or not(1, 2).

4.2 MATERIALS AND INSTRUMENTS

4.2.1 Materials

Table 4-1 List of materials used with their sources

Materials	Source
Cisplatin	Sun Pharma Advanced Research Centre, Vadodara.
Sodium caprylate	Sigma Aldrich
Dimethylformamide(DMF)	S.D.Fine Chemicals, Mumbai
Hydrochloric acid	S.D.Fine Chemicals, Mumbai.
Acetic acid Glacial	S.D.Fine Chemicals, Mumbai.
Potassium Bromide	S.D. Fine Chemicals, Mumbai, India.
Distilled Water	Prepared in Lab Distillation Assembly

4.2.2 Instruments

Table 4-2List of Equipment Used

Instruments	Source
Differential Scanning Calorimeter DSC-70	Shimadzu, Japan.
FTIR Spectrophotometer	Bruker, Japan.
Nanodrop	Thermofisher

4.3 METHODS

4.3.1 Selection and procurement of siRNA

siRNA selection was done based on concept of ABC efflux transporter protein knock down approach. Literature survey was done exhaustively to identify efflux protein responsible for fast removal of anti-neoplastic drug out of cancer cells. It revealed role of ABCC family for developing drug resistance due to fast efflux and

thereby diminished efficacy of drug therefore ABCC3 efflux protein was targeted for knock down approach using RNA interference technology and siRNA for ABCC3 protein was purchased from Santacruz biotechnology, USA. The siRNA is available as predesigned siRNA molecules as well as custom synthesized 21 mer or 27 mer duplexes. The knockdown efficiency of siRNA is claimed to be a function of siRNA sequence such as GC content, design and location of nucleotides at specific places and very much on the degree of siRNA target duplex stability. Therefore, all these parameters were studied in different predesigned sequences and a final choice was made. The complete characterization of the predesigned siRNA is provided in subsequent section. The purification of siRNA is done by desalting or HPLC. siRNA was procured as a ready to use HPLC grade duplex in the lyophilized form to be reconstituted in suitable quantity of 330 µl of the RNAase-free water provided

Purity determination of siRNA

As per manufacturer's instruction, The resuspension of the siRNA lyophilized duplex in 330 µl of RNAse-free water makes a 10 µM solution in a 10 µM Tris-HCl pH 8.0, 20 mM NaCl, 1 mM EDTA buffered solution. The efficacy of siRNA is function of proper design and choice of sequence. Although it cannot be quantitatively justified, confirming some basic design parameter will ensure optimal efficacy for the sequence selected. In most of the cases target site accessibility, free energy of structure equilibrium of siRNA-target hybridization lies as the base criteria for siRNA sequence selection.

The general guideline for selecting target sequences and siRNA sequences are:

1. The siRNA should target mRNA sequence 50-100 nucleotide downstream of ATG start codon.
2. Avoid stretches of 4 or more nucleotide repeats.
3. Avoid sequences which show certain degree of homology with other related or unrelated genes. It can be verified by running blast tool and noting similarity of sequences based on E value (expected value) which should be minimum.
4. Generally, highly functional siRNAs (having functionality more than 95%; ≥F95) have a G-C content that ranged between 36% and 52%.
5. The relative thermodynamic stability and ability to form internal hairpins can be estimated from the predicted melting temperatures (Tm) (1, 2). The formation of

internal hairpin structure can be avoided by choosing sequences with high Tm values. Duplexes lacking stable internal repeats are better silencers. Sorting the functional siRNA classes by Tm shows that no F95 duplexes exhibited Tm > 60°C or predicted hairpin structures.

siRNA was procured as a ready to use HPLC grade duplex in the lyophilized form to be reconstituted in suitable quantity of 330 µl of the RNAse-free water provided.Purity of the siRNA was checked by taking absorbance of 2 µl of 10 µM and 1 µM siRNA solution in the ratio of A260/A280 and A260/A230 using nanodrop by absorption spectroscopy which follows beer lambart law.

4.3.2 siRNA stability evaluation

Stability of working stock solution of siRNA was assessed after repeat sampling along with presence/absence of siRNA. Stability of siRNA was also assessed to evaluate effect of temperature and pH.

4.3.3 Preparation of cisplatin caprylate complex

Cisplatin (MW 300.1 g/mol) was complexed with caprylic acid (sodium salt) (MW 166.1 g/mol) in the molar ratios of 1:1, 1:2, and 1:3. Briefly, sodium caprylate was dissolved in water, and cisplatin was added gradually while stirring the solution at 60°C so as to have the maximum solubility of cisplatin (3). Heating was continued until the yellow color of cisplatin disappeared and white-colored complex formed. The resultant dispersion of complex was allowed to cool to room temperature(3).

4.3.4 Drug-Excipient compatibility studies

4.3.4.1 DSC Study:

DSC analysis was carried out using a Differential Scanning Calorimeter (DSC-60, Shimadzu, Japan) at a heating rate of 20°C per minute in the range of 25-300°C under inert nitrogen atmosphere at a flow rate of 40 ml/min(4). DSC thermogram of pure cisplatin, DPPC, PEG-PLA, and cisplatin caprylate were recorded to know melting pattern and confirm complexation reaction between cisplatin and sodium caprylate. Additionally, DSC thermograms were also recorded for Physical Mixture blend of HNCs excipients with cisplatin and Physical Mixture blend of HNCs excipients without cisplatin in the ratio of 1:1 to check the interference of drug with excipients.(4)(2)

4.3.4.2 FTIR Study:

IR-spectrum of cisplatin, sodium caprylate and cisplatin caprylate were measured in the solid state by preparing a Potassium Bromide (KBr) pellet to confirm absence of interference upon cisplatin caprylate complex formation in analytical method estimation.(5)The pure cisplatin was previously ground and mixed thoroughly with KBr, an infrared transparent matrix at 1:100 (sample KBr) ratio. The KBr pellets were prepared by applying 10-12 metric ton of pressure in a motorized pellet press (Kimaya engineers, India). The pellets were then scanned over a wavelength range of 4000-400 cm-1 and a spectrum was obtained by using a FTIR spectrometer-430 (Bruker). IR spectrums of physical mixture of excipients of HNCs with cisplatin caprylate were recorded in presence and absence of cisplatin caprylate.(6)

4.4 RESULTS AND DISCUSSION

4.4.1 Purity and concentration determination of siRNA

- ✓ Preparation of Primary stock solution:

  Resuspend the siRNA lyophilized duplex in 330 µl of RNAse-free water (provided by manufacturer) makes a 10 µM solution in a 10 µM Tris-HCl pH 8.0, 20 mM NaCl, 1 mM EDTA buffered solution

- ✓ Preparation of Secondary working stock solution:

  (A) 1 µM stock solution: Take 10 µl from above primary stock solution of siRNA in Nuclease free eppendrof tube and add 90 µl NFW which is equivalent to 100 nM/1000 µl or 1 pmol/µl or 13.3 ng siRNA.

- ✓ Purity and concentration determination

  As per manufacturer's instruction, The resuspension of the siRNA lyophilized duplex in 330 µl of RNAse-free water makes a 10 µM solution in a 10 µM Tris-HCl pH 8.0, 20 mM NaCl, 1 mM EDTA buffered solution. The optical density of 10 µM and 1 µM siRNA solutions A260/A280 were found to be 2.02 and 2.08 (Figure 4-1) respectively which is in accordance with the standard value of 2.0 for pure siRNA solution. The concentrations of solutions analyzed by nanodrop were 127.64 ng/µl and 12.06 ng/µl for 10 µM and 1 µM respectively confirming the results in agreement with the theoretical calculations of by considering siRNA molecular weight i.e. (1 nmol siRNA= 13.3 µg siRNA or 1 pmol= 13.3 ng siRNA). The average molecular weight of a 21 mer siRNA duplex is 13,300

g/mol. Conformation of molecular mass of individual strand i.e. sense strand and anti-sense strand were determined by matrix assisted laser desorption ionization-time of flight (MALDI-ToF) technique. The molecular mass of sense strand and anti-sense were reported as 6575 and 6725 Da.

Figure 4-1 Nanodrop spectra siRNA purity and concentration determination

Table 4-3 Purity and concentration determination of siRNA using nanodrop

Items	Results
OD260	0.327
OD280	0.270
OD230	0.182
OD320	0.025
Pathlength	0.719
Dilution	1.0
OD 260/280	2.08
OD 260/230	1.93
Nucleic acid concentration	12.06 ng/µl

4.4.2 siRNA stability evaluation

As siRNA is highly unstable, the stability of residual siRNA working solution after repeat sampling was assessed. The RNase contamination during sampling can degrade remaining stock, which may result in lower siRNA in subsequent sampling. Therefore, stability of sampled secondary stock against fresh sample from primary

stock was checked. Figure 4-2 shows that the siRNA was intact after repeat sampling and remains stable.

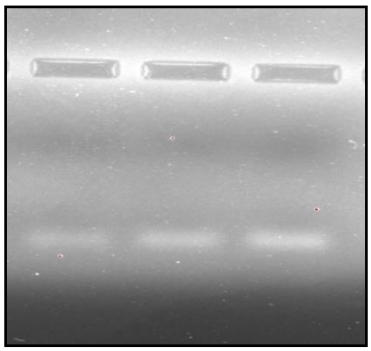

Figure 4-2siRNA stock solution stability

(all lanes 4 pmol)

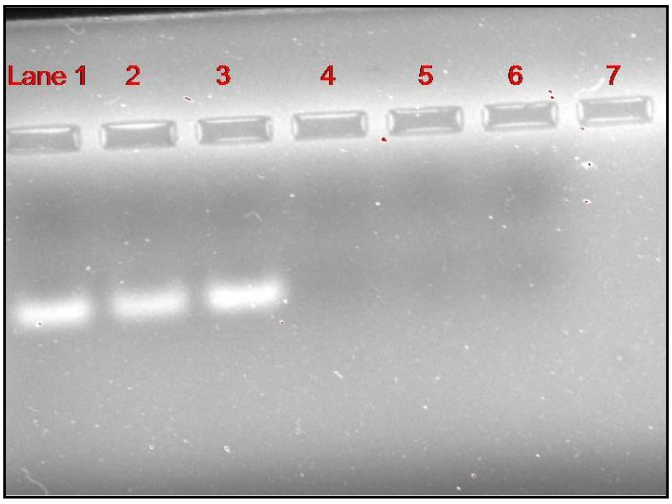

Figure 4-3 Gel electrophoresis of siRNA

(lane 1, 2, 3: 10 pmol, lane 4,5: siRNA+RNase; lane 6: DEPC treated water lane 7: NFW)

From the Figure 4-3, it was concluded that RNAse completely degraded siRNA (lane 4, 5). Thus, siRNA molecules must get RNase free exposure and hence, DEPC treatment was used to remove RNAse from all materials used for materials.

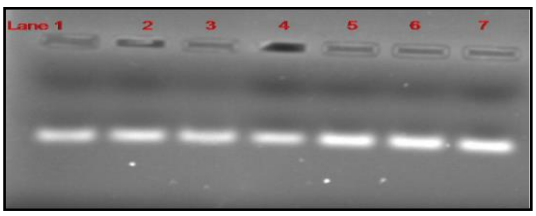

Figure 4-4 Effect of temperature and pH on siRNA

(Lane: 1: 25 °C, 2: 60 °C, 3: 40 °C, 4: 30 °C, 5: pH 6.5, 6: pH 7.5, 7: pH 8.5)

siRNA is prone to degradation with heating and the same was observed by incubating the siRNA at different temperature for fixed period of time i.e. 30 min. **Figure 4-4** showed that more than 40 % of siRNA (band densitometry method) was degraded at 60 C hence temperature above 50 C must not be used during processing any operation in formulation where siRNA is involved. **Figure 4-4** shows the pH stability of siRNA at different pH. It showed that siRNA was stable at weakly alkaline condition (pH 7.5) and weakly acidic condition (pH 6.5).

4.4.3 Preparation of cisplatin caprylate complex

Cisplatin though being water soluble molecule, has aqueous solubility of around 2.5 mg/ml only. Thus, its entrapment in either the aqueous or the bilayer of lipid vesicles is limited.To improve the lipid solubility, one of the strategies used is complexation with ligand molecules. This would also lead to improvement in the partitioning of the drug towards the amphiphilic core of the copolymer. The reaction of cisplatin with sodium caprylate is of coordination complexation type wherein the positive charge attained by the drug in aqueous solution interacts with the negatively charged ligands to form complexes. To aid the reaction, temperature of the solution was increased to 60°C which increased the kinetic energy of the molecules enhancing solubilization of drug and further pH of the medium chosen was close to the pKa which ensured higher ionization of the parent drug. Herein upon completion of reaction, thesynthesized cisplatin-caprylate complex was obtained as a fine white dispersion. The dispersion was observed visually under optical microscope to confirm absence of yellow colored precipitates which indicated incomplete complexation (Table 4.4). Thus, for the ease of processing and subsequent use,1:1 ratio was selected due to white colored uniform precipitates.

Table 4-4 Cisplatin caprylate complex formation at different molar ratio

Cisplatin & caprylate molar ratio	Complex formation	Remarks
1:0.5	-	Yellow precipitates of cisplatin
1:1	++	White uniform dispersion complex
1:2	+	Aggregates
1:3	+	Aggregates

4.4.4 Drug-Excipient compatibility studies

4.4.4.1 DSC

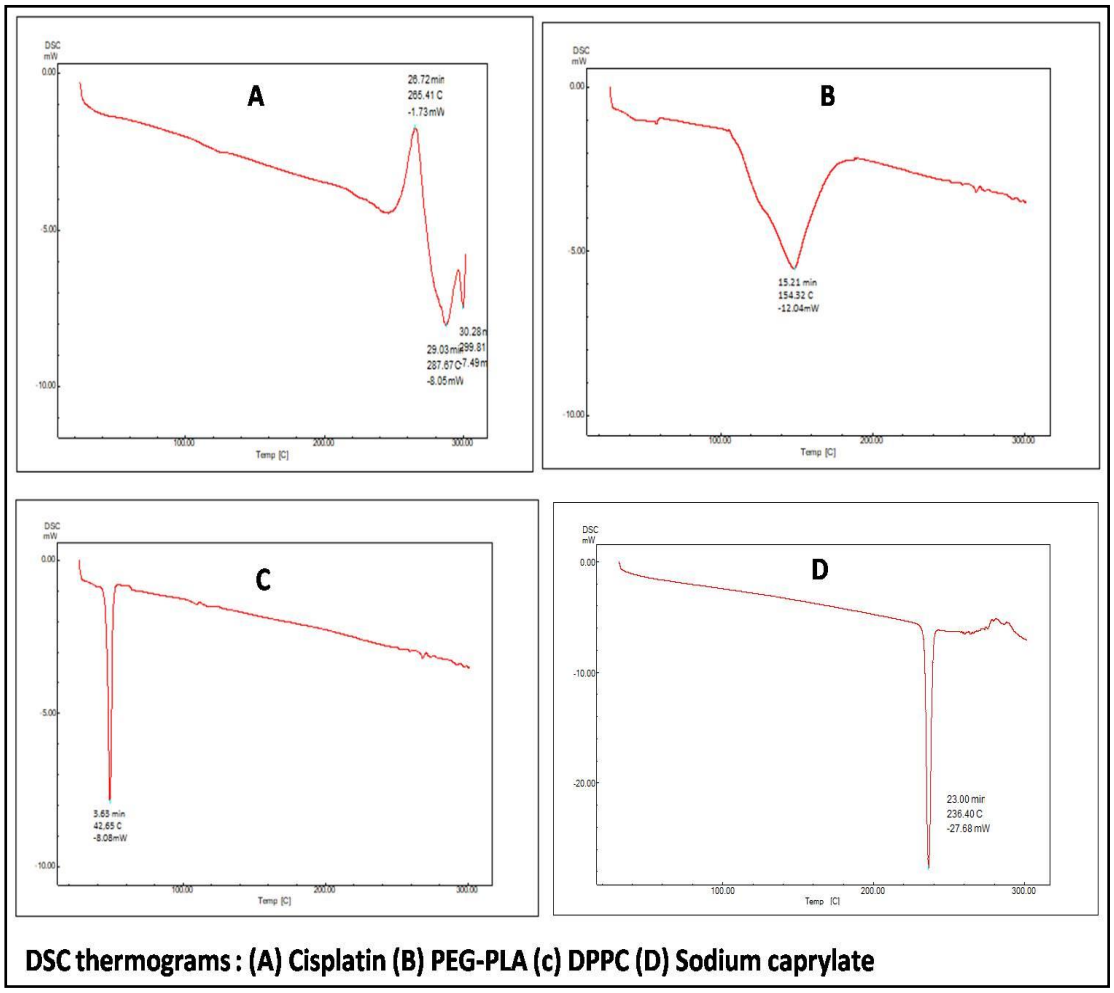

Figure4-5 DSC thermogram-I

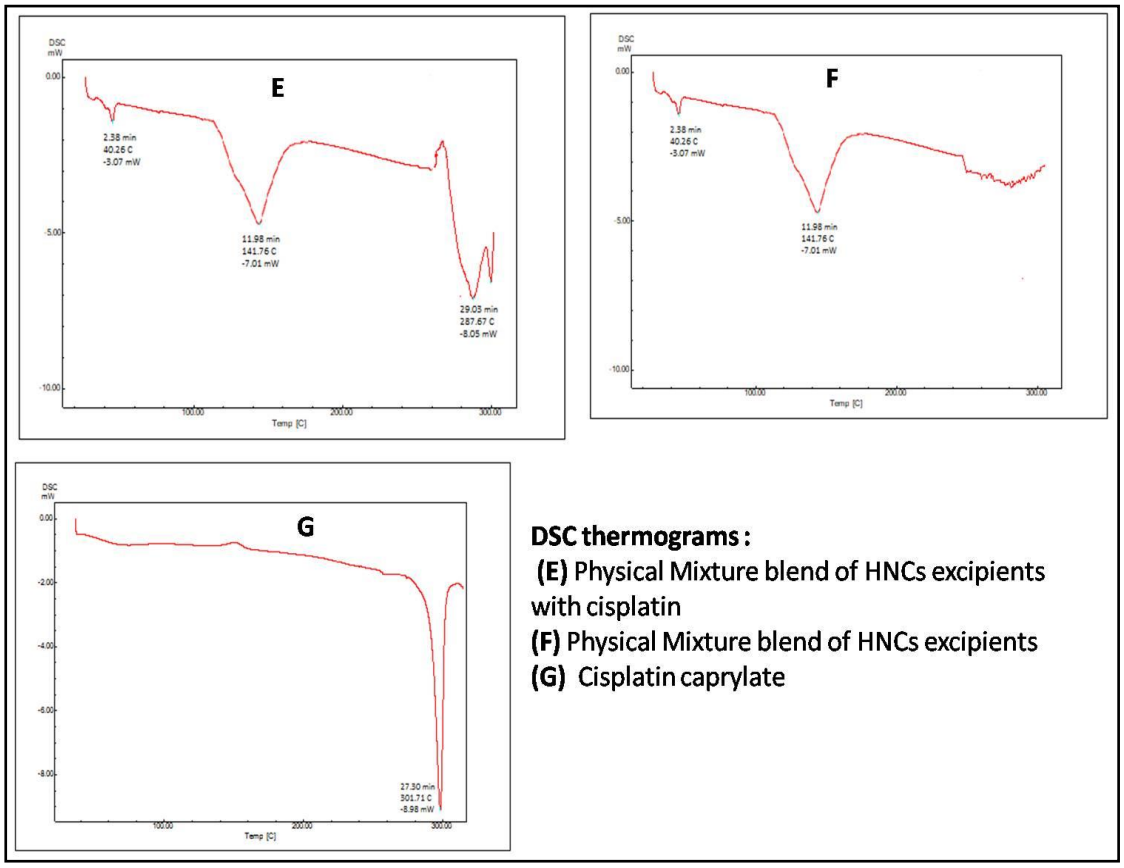

Figure4-6 DSC thermogram-II

Differential Scanning Calorimetry (DSC) is technique in thermal analysis to observe endothermic processes (chemical degradation, Melting) and exothermic processes (Crystallization and oxidative decomposition). It reveals valuable information in pre-formulation studies as it shows the occurrence of possible drug-excipient or excipient-excipient interaction in dosage form. DSC thermogram of cisplatin caprylate showed sharp endothermic peak at 300 °C (Figure4-6 G) distinct from the melting point of either cisplatin (Figure4-5 A) (~287°C) or sodium caprylate (Figure4-5 D) (~ 118°C). This also confirms the melting point range (295°-305°C) observed using capillary method. Figure4-5 B shows DSC thermogram of PEG-PLA which shows broader peak at 152 °C corresponding to the melting point of PEG-PLA having mixed crystalline-amorphous property. Figure4-5 C gives DSC thermogram of DPPC showing sharp endothermic peak at 40.91 C conforming glass transition temperature 41 C. Figure4-6E and Figure4-6F shows the DSC Thermogram of Physical Mixture blend of HNCs excipients with cisplatin and without cisplatin respectively. There was no change in position endothermic peak of drug therefore it can be confirmed that the

drug and excipients are compatible with one another. DSC thermograms of sodium caprylate and cisplatin-caprylate complex confirmed the complexation reaction cisplatin and caprylate.

Table 4.5comparison of endothermic peak of DSC

Material	**Endothermic peak in DSC at temperature (°C)**
Cisplatin	287°C
Sodium Caprylate	118°C
Cisplatin Caprylate	300°C
PEG-PLA	152°C
DPPC	40.91°C
Physical mixture of drug and excipients	40.26°C; 141.76°C; 287.67°C
Physical mixture of excipients	40.26°C; 141.76°C

4.4.4.2 FT-IR

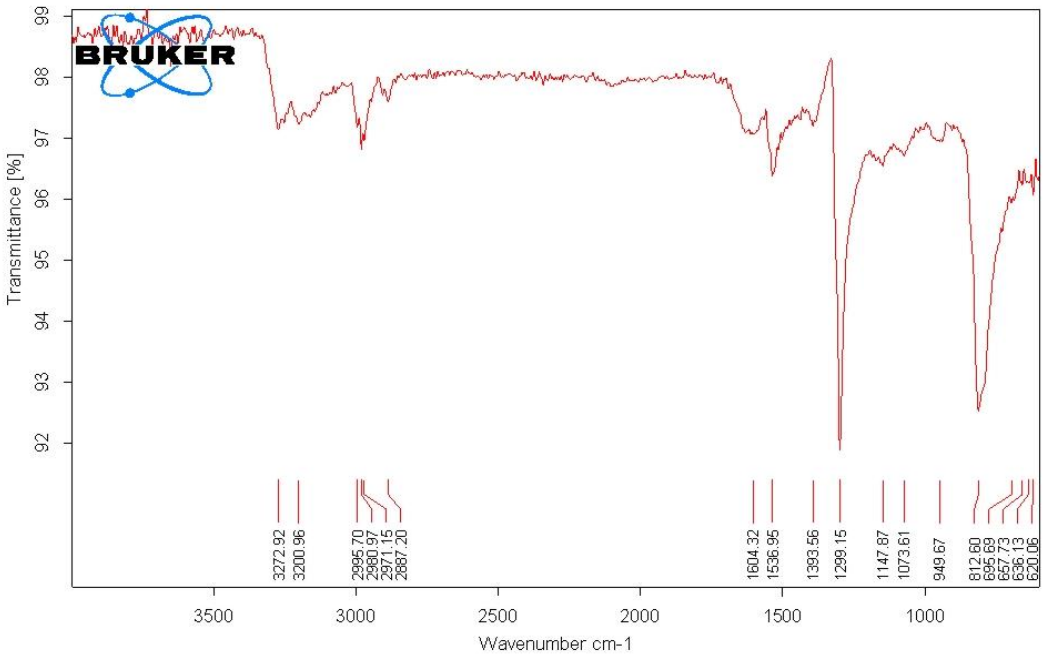

Figure4-7IR spectrum of cisplatin

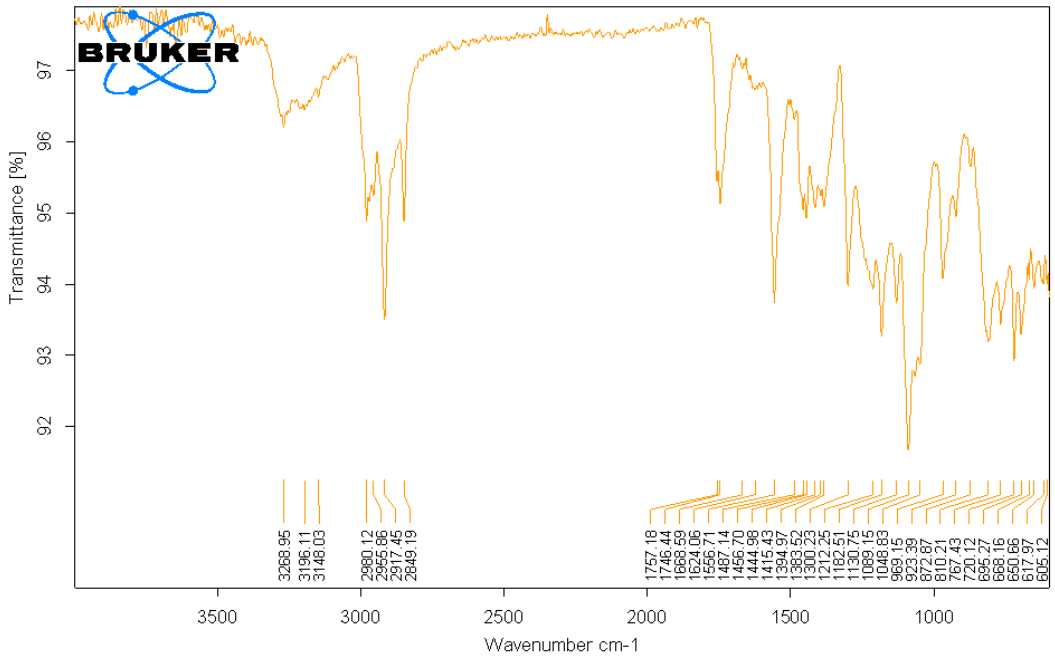

Figure4-8 IR spectrum of physical mixture of HNCs components with cisplatin

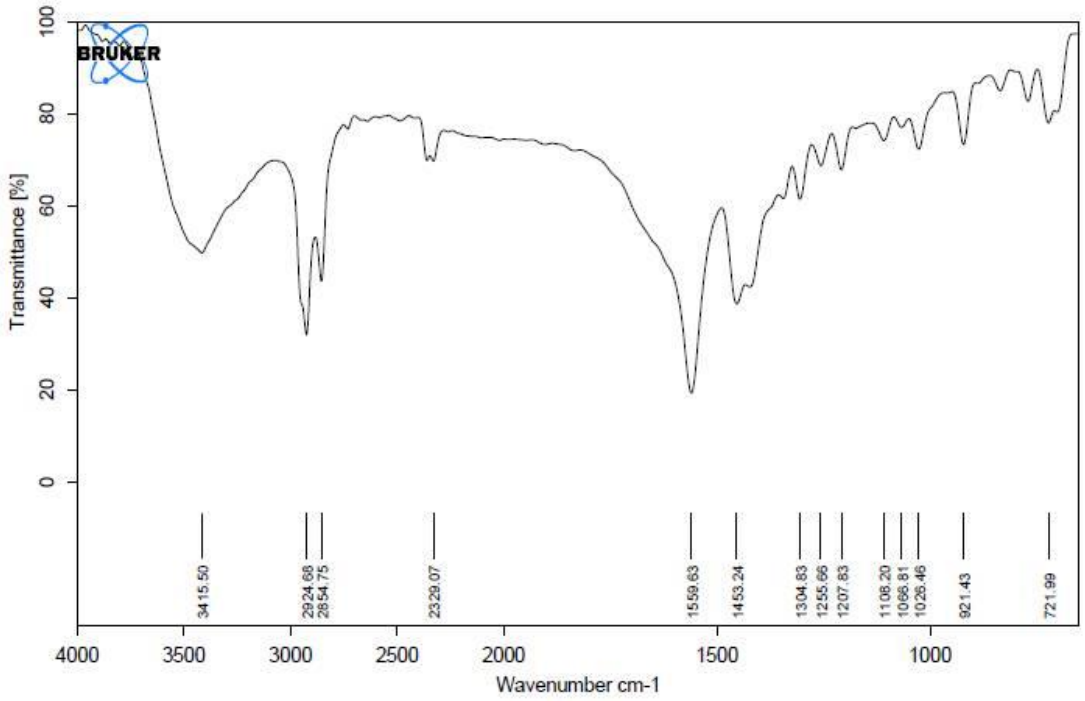

Figure4-9 IR spectrum of Sodium caprylate

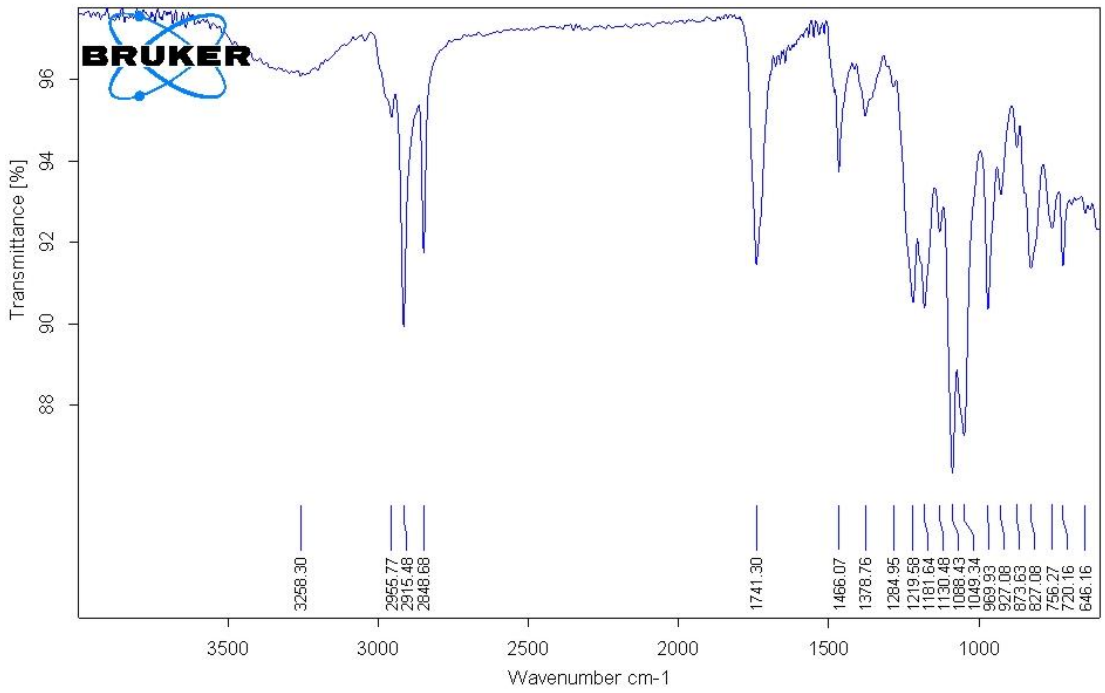

Figure4-10 IR spectrum of physical mixture of HNCs components

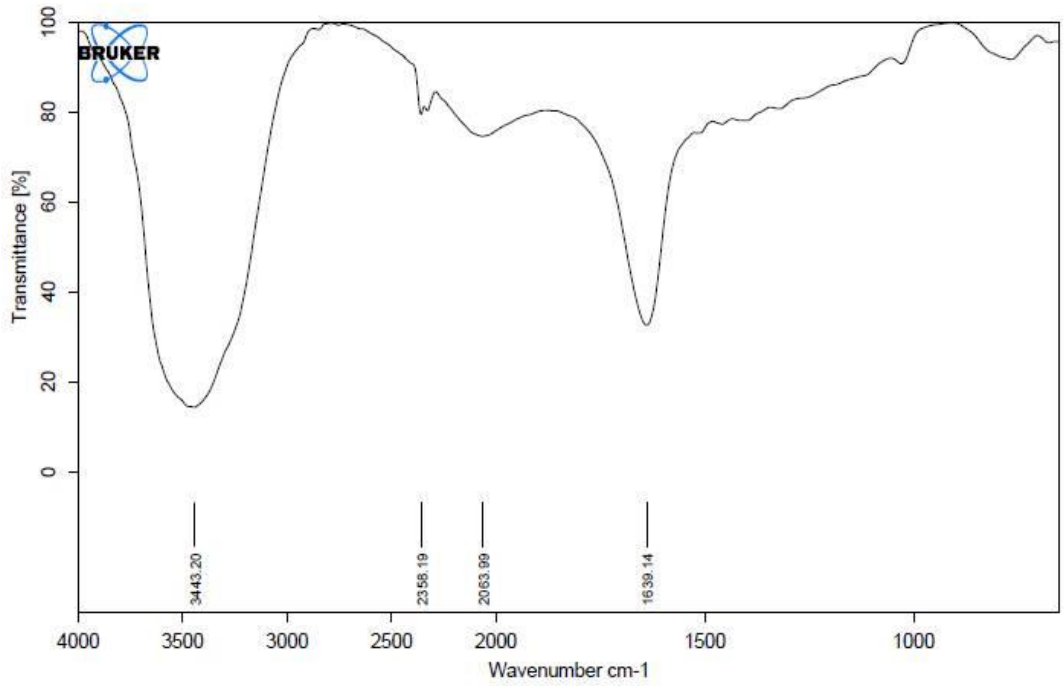

Figure4-11 IR spectrum of cisplatin caprylate

The IR Spectrum of cisplatin is shown in Figure4-7. The characteristic bands were identified. IR of physical mixture (**Figure4-8**) (Cisplatin+PEG-PLA+DPPC+DOTAP+DSPE PEG2000), sodium caprylate (Figure4-9) was taken to

check compatibility of drug and excipients. Comparison of FTIR spectra(Figure4-7 and **Figure4-8**) cisplatin caprylate against the individual spectra of cisplatin and sodium caprylate shows marked changes in few characteristic peaks. The peak of carbonyl group of caprylate at 1560 cm^{-1} was shifted to 1640 cm^{-1}. Furthermore, the disappearance of characteristic peaks at 2105 cm^{-1} and 2623 cm-1 for cisplatin in the complex indicated both the hydroxyl group in the aqua species of cisplatin were involved in complex formation. These shifts support the complex formation via carboxylate group of caprylate with cisplatin. It was also observed that characteristics peak of amino group in the spectral range from (3600 cm^{-1} to 3300 cm^{-1}) of cisplatin was intact in complex form.(Figure4-11 and Figure4-9). Cisplatin caprylate complex formation wouldlead to increase in the lipophilicity of cisplatin,which could help in partitioning of the drug more towards the lipophilic bilayer as well as in the polymeric core, thereby increasing entrapment of cisplatin in HNCs which consist of lipidic as well as amphiphilic components.

Table 4-6 Comparison of IR peaks

Material	Cisplatin	Sodium Caprylate	Cisplatin Caprylate	Physical mixture of excipients	Physical mixture of drug and excipients
Characteristic Peaks in FTIR (cm-1)	-	3415.50	3443.20	-	-
	3272.92	-	-	3258.3	3268.95
	3200.96	-	-	-	3195.11
	-	-	-	-	3148.03
	-	-	-	-	2980.12
	2995.70	-	-	2955.77	2955.86
	2980.97	2924.68	-	2915.48	2917.45
	2971.15	2854.75	-	2848.68	2849.19
	2887.20	2329.07	2358.19	-	-
	-	-	2063.99	-	-
	-	-	-	1741.30	1757.18
	-	-	-	-	1746.44
	-	-	-	-	1688.59
	1604.32	-	1639.14	-	1624.06
	1536.95	1559.63	-	-	1556.71
	-	1453.24	-	-	1487.14
	-	-	-	1466.07	1456.70
	-	-	-	-	1444.98
	-	-	-	-	1415.43
	1393.56	-	-	-	1394.97
	-	1304.83	-	1378.76	1383.52
	1299.15	1255.66	-	1284.95	1300.23
	-	1207.83	-	1219.58	1212.25
	1147.87	-	-	1181.64	1182.51

	-	1108.20	-	1130.48	1130.75
	1073.61	1066.81	-	1088.43	1089.15
	-	1026.46	-	1049.34	1048.83
	949.67	-	-	969.93	969.15
	-	921.43	-	927.08	923.39
	-	-	-	873.63	872.87
	812.63	-	-	827.27	810.21
	-	-	-	756.27	767.43
	-	721.99	-	720.16	720.12
	695.69	-	-	-	695.27
	657.73	-	-	-	668.16
	636.13	-	-	646.16	650.66
	620.06	-	-	-	617.97
	-	-	-	-	605.12

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