Research Article

Preformulation considerations of Natamycin and development of Natamycin loaded niosomal formulation

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Abstract

Background: Natamycin (NAT) is a broad-spectrum polyene antifungal agent employed in the treatment of fungal keratitis, blepharitis, and conjunctivitis. It is specially used against filamentous fungi due to its better safety profile, and transcorneal penetration potential. **Objective:** To assay the physicochemical properties of natamycin and development of its niosomal formulation. **Material and Methods:** The natamycin was characterized for its meting point, solubility profile and partition coefficient, which were almost similar as standard. Natamycin was insoluble in almost organic and inorganic solvents. Fourier Transform Infrared (FTIR) and UV-Visible spectroscopy were also performed and the spectra for both spectroscopies were almost superimposable with previously reported spectra. The Differential Scanning Calorimetry (DSC) study gave an idea about the compatibility of natamycin with other formulation additives. Thin Film Hydration (TFH) method was employed for the development of natamycin loaded niosomal formulation and it was characterized for vesicle size, zeta potential, % entrapment efficiency (% EE) and percentage drug release up to 12 hrs. **Results and conclusion:** In preformulation studies the absorption maxima for drug was found to be 317 nm. The drug is insoluble in almost all solvents, but it was showing solubility only in acetic acid, acidic methanol and mixture of chloroform-methanol (3:2). The melting point of drug was found to be 290-295 °C, which was indicating the thermal stability of drug during entire range of temperatures for formulation development. The FTIR spectrum of the drug is almost similar to the standard which indicates the purity and stability of drug. The vesicle size, zeta potential and % EE of the developed niosomes were found to be 968±8.24 nm, 22.81±2.19 mV and 78.61±3.09 % respectively. The % drug release was found to be 87.66±4.32 % in 12 hrs. The results of all parameters are acceptable and can be utilized for further studies.

Keywords: Natamycin, Niosomes, physicochemical characterization, solubility, UV spectroscopy, FTIR spectroscopy

Introduction

Mycotic keratitis, commonly known as fungal keratitis, accounts for approximately 1-44% of all cases of microbial keratitis, depending upon the geographic location (Garg, 2012). Overall, it is more common in tropical and subtropical areas. The genera that commonly cause infection of the cornea include *Fusarium, Aspergillus, Curvularia, Bipolaris*, and *Candida* (Qiu et al., 2015). Most of the currently available antifungal medications have limitations, such as poor bioavailability and limited ocular penetration, especially in cases with deep-seated lesions (Ansari et al., 2013). To overcome these limitations a number of newer antifungal agents and drug delivery techniques are being tried (Chang and Chodosh, 2011). The antifungal agents used for the treatment of fungal keratitis include three classes: polyenes, triazoles, and echinocandins. Natamycin is a tetraene polyene which has been regarded as the most important agent in the management of fungal keratitis. It acts by binding with ergosterol, which is an essential component in fungal cell wall, and blocks fungal growth. Natamycin is the only antifungal medication approved by U.S. Food and Drug Administration (Arora et al., 2011).

Niosomes are vesicular system developed with the help of surfactants. Niosomes depict quite resemblance to liposomes as per the structure and physical properties are concerned (Kaur et al., 2004; Paecharoenchai et al., 2014; Jain and Jain,

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2017). Vesicles comprising of one or more surfactant bilayers confining aqueous spaces are called non-ionic surfactant vesicles or niosomes. Because of the various advantages offered by the niosomes over liposomes such as better skin permeation potential, higher chemical stability, sustained release characteristic (Uchegbu and Florence, 1995; Kaur et al., 2004), quite economic due to the ease of access of the starting ingredients, biocompatibility and non-immunogenicity (Kaur et al., 2004) and cost effective handling procedures like no specific requirement for storage in freezer and use of nitrogen for the preparation, the niosomal formulations are grabbing the attention of researchers.

The major objective of this research was to characterize natamycin in context of physicochemical parameters (solubility, meting point and partition coefficient) useful for the development of natamycin loaded niosomes. The Infrared spectroscopy, UV-spectroscopy and HPLC analysis were performed for the drug. The differential scanning calorimetry was done for the evaluation of drug-excipients interaction. The prepared niosomes were characterized for vesicle size, zeta potential and % EE and *in vitro* drug release.

Materials and methods

Materials

Natamycin was procured as gift sample from AKUMS Drugs & Pharmaceuticals Ltd. (Haridwar, UK, India). Cholesterol (chol) was procured from Himedia, Mumbai, India. Span 60 (sp60) and Sodium hydroxide were purchased from CDH (Central Drug House; New Delhi, India). Sodium chloride and acetone were purchased from Fisher scientific India. Cellulose acetate membrane filter (syringe filter) was purchased from Chromatopak. Methanol (Merck Life Science Pvt. Ltd., Mumbai; India), chloroform (Merck Life Science Pvt. Ltd., Mumbai; India), Ultrapure water (Millipore, Bedford, MA) was used throughout the studies. All other chemicals were of the highest grade commercially available.

Physicochemical characterization of natamycin

The development of an efficient dosage form is usually a long and complex process. Prior to dosage form development, it is absolutely necessary that certain fundamental properties of candidate drug molecules are to be determined. This helps the formulator to generate useful information that could help in the successful and productive development of an efficient dosage form. The preformulation studies were performed to gain the sound knowledge about the drug. Preformulation may be described as authenticating the drug by the determination of their physical and chemical properties, which are considered as important factor in the formulation of a stable, effective and safe dosage form. Several physicochemical parameters were evaluated for NAT. The figure 1 shows diagrammatic representation of physicochemical parameters for natamycin.

Melting Point determination

Determining the melting point of a compound is one of the way to test verify that the substance is pure. A pure substance generally has a melting range (the difference between the temperature where the sample starts to melt and the temperature where melting is complete). Impurities tend to depress and broaden the melting range so the purified sample should have a smaller melting range. Special glass capillary was selected for melting point apparatus (Digital Melting Point apparatus JSGW). Open end of the capillary was filled with the sample of drug. Gently glass capillary was tapped on a hard surface i. e. table, so that the drug was moved down to the very bottom of the glass capillary. Melting Point was measured by inserting the glass capillary into the melting point apparatus. The apparatus was heated with a rate of 10

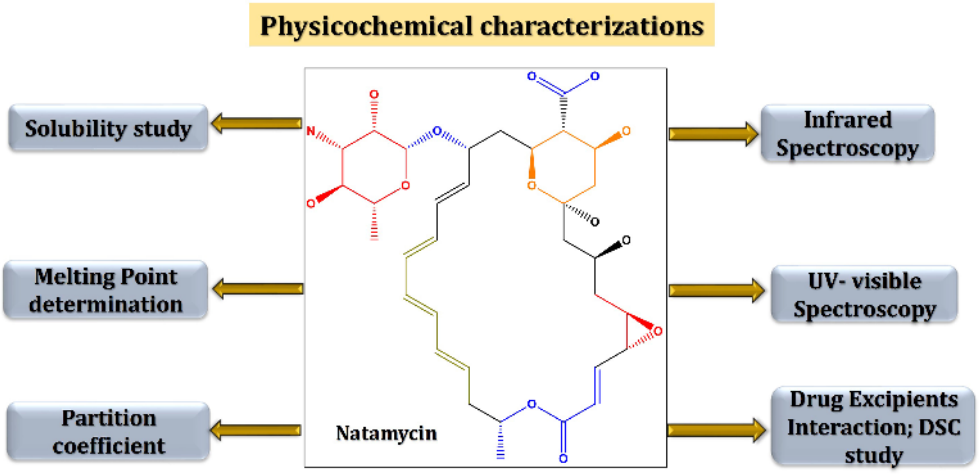

Figure 1. Summary of physicochemical characterization of natamycin

°C/min until it is close to the expected melting point. Then heating rate was decreased with 1°C/min and the sample was closely observed. The temperature was recorded when the sample started to melt and when it was completely molten. Whole protocol was performed in triplicate (Paseiro-Cerrato et al., 2013).

Determination of absorbance maxima (λ_{max}) and preparation of standard curve

Natamycin was accurately weighed (10 mg), moistened with 0.625 ml of water. The resulting wet mass was kept in dark place for a while and then the volume was made up to 25 ml with mixture of methanol-glacial acetic acid (1000:1) in 25ml volumetric flask. Then from that stock solution 1ml was pipetted out into a 25 ml volumetric flask and volume was made up to the mark with methanol-glacial acetic acid mixture. The resulting solution was scanned between 200-400 nm by using UV- Visible spectrophotometer [Shimadzu, Japan]. The λmax was found to be 317 nm (Figure 1).

This stock solution of 100 µg/ml was used to prepared aliquots of 0.2ml, 0.4ml,…….up to 2ml and volume was made up to 10ml in a 10 ml volumetric flask with the mixture of Methanol : Glacial Acetic Acid (1000:1). These aliquots were analysed at λmax 317 nm by using UV Visible spectrophotometer (Shimadzu, Japan). The standard curve was plotted between absorbance and concentration at 317 nm (Figure 2).

Solubility studies

Solubility is an essential and extensively studied preformulation parameter. Such information is important to the formulator, for it enables him to select the best solvent medium for a drug, identify and overcome certain challenges that arise in the formulation of pharmaceutical solutions, and, furthermore, estimate the level of all known and significant impurities (Allen and Ansel, 2013). **Solubility is defined as maximum amount of solute that dissolves in a solvent under suitable condition of equilibrium, at a specific temperature.** In qualitative terms, it is defined as spontaneous interaction of two or more substances to form an identical molecular dispersion. Natamycin is amphoteric in nature and has solubility in very few solvents. The drug sample was quantitatively and qualitatively tested for its solubility in various polar or non-polar solvents (Badhani et al., 2012; Brik, 1981). The quantitative estimation of drug sample was performed by taking excess of drug in 1ml of particular solvent. Drug and solvent were placed in a tightly closed vial and allowed to shake in wrist shaker for 12 hrs and then the vial was kept aside for a while. The supernatant was collected after settling of undissolved solute and solubility of drug was determined spectrophotometrically (λ_{max}=317 nm). Solubility profile of natamycin is shown in table 1.

Partition coefficient

The partition coefficient of drug was determined by traditional shake flask method using n-octanol or chloroform as organic phase and water or phosphate buffer as aqueous phase. The organic and aqueous phases were equilibrated with each other by shaking them together in wrist action shaker for equilibration (about 12 hrs). After this both phases were separated and aqueous phase was centrifuged after equilibration in order to remove small n-octanol droplets which was the result of the emulsion formation during shaking. A known volume (2 mL) of aqueous phase containing drug was transferred into 2 mL of organic phase. The mixture of both phases was placed into a 5 mL tube, and allowed to stir for 24 hrs on an orbital shaker at a temperature of 37.0 ± 0.5 ºC, in triplicates. Samples were withdrawn, centrifuged (4000 rpm, 15 mins) and diluted suitably with phosphate buffer pH 7.2. The sample was analyzed spectrophotometrically at 317 nm. The partition coefficient was calculated using the formula:

$$\text{Partition coefficient} = \frac{C_{oil}}{C_{aqueous}}$$

Where, C_{oil} and $C_{aqueous}$ were representing amount of the drug in oil phase and aqueous phase respectively.

The partition coefficients of NAT in different systems are showing in the table 2.

FT-IR analysis

Drug sample was vacuum dried for 12 hrs before this analysis. The FTIR spectrum of drug; natamycin was obtained using Bruker Tensor-37, FTIR. The drug in powder form was directly placed onto the crystal and scanned between wave number 4000^{-1}– 600 cm^{-1} in a FTIR spectrophotometer (Bruker Tensor-37, FTIR) (Smith, 2011). The characteristic peaks are reported in figure 4.

Drug excipients compatibility

The efficacy of drug delivery system depends not only on the active principles and production processes, but also on the performance of the excipients. The magnitude of effect is depending upon physicochemical properties of drugs as well as quantity and quality of excipients used. Although often regarded as 'inert', excipient can in fact readily interact with drugs. The evaluation of drug-excipient compatibility is therefore an essential aspect of any preformulation study. Incompatibility between drug and excipient can alter stability and bioavailability of drugs, thereby, affecting its safety and/or efficacy. Study of drug–excipient compatibility is an important process in the development of a stable dosage form. Drug–excipient compatibility testing at an early stage helps in the selection of excipient that

increases the probability of developing a successful dosage form. A number of techniques can be used to indicate the drug/ excipient interaction. Differential scanning calorimetry (DSC) is one of the well-developed techniques used in the detection of incompatibilities in drug/ drug and drug/excipient interactions. Differential thermal analysis (DTA) is similar to DSC in many respects and analogous information about the same range of thermal events can be observed. Other non-thermal methods that have been used to indicate the drug/excipient interactions include Infrared spectroscopy, optical microscopy and HPLC studies.

Differential scanning calorimetry (DSC) represents a thermo analytical method that measures the difference in the thermal energy required to increase the temperature of a sample and a reference with well-defined heat capacity as a function of the temperature.

Ideally, both the sample and the reference are kept at identical temperature, while the temperature increases linearly as a function of time. As soon as the sample undergoes a physical transformation; either exothermic or endothermic, the heat flow to the sample will be decreased or increased relative to the reference, thus, resulting in a DSC signal. DSC is widely used to study the drug-excipient interaction as well to detect the thermal properties of drug (s) as well as the materials of formulation.

The DSC study was carried out for natamycin and physical mixture of drug and excipients. DSC analysis was done for drug separately and then physical mixture (Natamycin plus Cholesterol plus soya PC) was analyzed. These studies were conducted over a temperature range between 30 and 300°C using a differential thermal analyzer (NETZSCH STA 449 F1, Leading Thermal Analysis). Thermographs were obtained by heating 3-5 mg of sample in crimped aluminium pans at heating rate of 10°C/min, from 0°C to 300°C and sample analysis was performed under nitrogen pumping (flow rate: 20 mL/min) (El-Ridy et al., 2015; Clas et al., 1999). Data were analyzed to obtain onset temperature (T onset); the peak temperature (T peak) and the end set temperature (T end set) of peak. DSC of NAT and Physical mixture are shown in figure 5.

Preparation of Niosomes

Various methods are available for the formulation of surfactant based vesicular system. Niosomes were formulated using modified thin film hydration method (Baillie et al., 1985; Azmin et al., 1985; Udupa et al., 1993). Briefly, different amounts of NAT (30 mg), span 60 (66 mg)Chol (24 mg) and DCP (5%w/w) were weighed into a long-necked round-bottom flask (50 mL) and dissolved in 10 mL of chloroform–methanol mixture (3:2, v/v). The organic phase was gradually evaporated at 50 °C under vacuum, using a Rotary Evaporator (Rotary vacuum evaporator quickvap, Superfit Rotavapor, India) at 60 rpm to get a thin dry film on the inner wall of the flask (Al-Mahallawi et al., 2014). The dried thin film was then hydrated with phosphate buffer (pH 7.2) using rotary evaporator at 50 ºC. The obtained dispersion was sonicated (bath sonicator, Elmasonic S40, Elma, Singen, Germany) for 1 min to obtain small size vesicles (Verma et al., 2019; Jain and Jain, 2016b). Niosomes were purified to remove off unentrapped drug and unreacted matter by two step ultra-centrifugation process (20,000×g, 4 °C, and 30 min) and washed twice with phosphate buffer (pH 7.2).

Characterization of Niosomes

Vesicle size, polydispersity index and zeta potential

Vesicle size (z-average), size distribution (polydispersity index) and ZP of developed Niosomes were evaluated using NanoPlus-3 (Version 5.01, Micromeritics Instrument Corporation, Particulate Systems, Norcross, GA, USA) by Photon Correlation Spectroscopy (PCS). The formulation was diluted to 1:9 *v/v* with ultrapure water. The formulation was placed into the cuvette and observed at 90° fixed angle. The ZP of Nios was determined using Helmholtz-Smoluchowsky equation from their electrophoretic mobility at 20 V/cm field strength and 50 μs/cm^4 conductivity (Khalil et al., 2017). The results are depicted in table 3.

Entrapment efficiency (%EE)

Ultra-centrifugation method was employed for the determination of amount of encapsulated natamycin in vesicular systems. Niosomal suspension (1 mL) was centrifuged at 20,000 rpm for 1 hour using a cooling centrifuge (C-24, Remi instruments, India) at 4 °C. The niosomal vesicles were separated and washed two times with 1 mL PBS. The supernatant was separated each time and assayed by HPLC method using Phenomenex Luna C-18(2) column (4.6 X 250 mm, dp = 5 μm with a mobile phase of methanol: water: acetic acid (12:8:1v/v/v), at 1mL/min flow rate at 303 nm [Shimadzu (Kyoto, Japan) HPLC equipment, Model-SPD-M20A, fitted with a; Hyderabad, India)]. The free drug was subtracted from the total drug added into the formulation in order to calculate the entrapped drug (table 3) (El-Badry et al., 2015). The EE (%) was determined by following formula:

$$\%EE = \frac{(\text{Total drug taken}) - (\text{drug found in supernatant})}{\text{Total drug taken}} X\ 100$$

Drug release

The release of natamycin from niosomes was determined using the dialysis bag method. An accurately measured amount of niosomal formulations (equivalent to 30 mg drug), suspended in 5 ml phosphate buffered saline (pH 7.2) was taken in dialysis bag (HiMedia dialysis membrane

12,000–14,000 Mwt cutoff). This bag was kept in 100 mL ATS (Artificial Tear Solution) (pH 7.2) at 37±0.5°C with continuous slow magnetic stirring with 20-25 rpm (Model; Tarsons SPINOT Magnetic Stirrer Hot Plate with dimension: 12 x 11 x 13 cm, 19 x 11 x 21 cm). Samples from release medium were periodically withdrawn for 12 hrs and immediately replaced with fresh ATS (pH 7.2). Samples were analysed by HPLC method using Phenomenex Luna C-18(2) column (4.6 X 250 mm, dp= 5 µm with a mobile phase of methanol: water: acetic acid (12:8:1v/v/v), at 1mL/min flow rate at 303 nm [Shimadzu (Kyoto, Japan) HPLC equipment, Model-SPD-M20A, fitted with a; Hyderabad, India] (Tavano et al., 2013). Finally, cumulative % natamycin release was calculated from the obtained data and is shown in the figure 6.

Statistical analysis

All results were expressed as mean ± SD and the statistical analysis was done with one-way ANOVA with Tukey–Kramer multiple comparison post-test using NCSS 2007 Version 07.1.14 (Utah, USA). *In vitro* drug release kinetics model fitting was performed using Sigma Plot for Windows Version 11.0 (wpcubed GmbH, Germany). A difference with $p \leq 0.05$ (i.e. 5% level of significance) was considered to be statistically significant.

Results and discussion

There were two main objectives of the drug characterization. The first one was to compare the experimentally determined properties of the drug with the one that are reported in the Pharmacopoeia in order to authenticate the drug sample and the other objective was to perform the preliminary studies of properties of drug that can be helpful during further studies and experiments.

Physicochemical characterization of natamycin

Melting point

The melting point of natamycin was found to be 290-295 ºC. It darkens at about 200°C and "melts" under vigorous decomposition. The result of melting point indicated that the drug was stable in the entire formulation temperature range.

Determination of absorbance maxima (λmax) and preparation of standard curve

UV scan of natamycin was performed in in methanol-glacial acetic acid (1000:1) mixture at 317 nm λmax (Figure 2) using UV–Visible spectrophotometer Shimadzu, Japan. These values were quite similar to the previously reported literatures.

The standard curve of NAT was prepared in mixture of methanol-glacial acetic acid (1000:1). The absorbance data of the drug was obtained subjected to linear regression at 317 nm and the obtained absorbance data was subjected to linear regression (figure 3). The correlation coefficient was found to be 0.999 which indicated that Beer's law is obeyed within the concentration range (2-20µg/ml) that was used in the experiment.

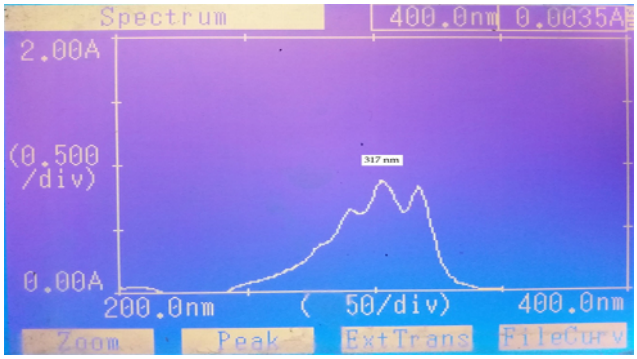

Figure 2. UV Scan of Natamycin in Glacial acetic acid: Methanol (1:1000) (sample)

Solubility studies

The solubility of natamycin was determined in different media which would be used in the formulation development. It was insoluble in chloroform, acetone and 95% ethanol as well as in water, acidic (pH 3.6) and basic (pH 7.4) buffer. Natamycin was sparingly soluble in methanol. It was freely soluble in acidified methanol (Glacial acetic acid: methanol; 1:1000) and very soluble in chloroform: Methanol (3:2) mixture (Table 1).

The results from solubility analysis were showed the solubilizing behaviour of drug in different solvents. The natamycin has amphoteric nature due to the presence of various groups in its chemical structure, which leads to its insolubility in many organic and inorganic solvents.

Partition coefficient

Partition coefficient estimation of the drug was performed in two different systems i. e. chloroform: PBS (pH 7.4) and n-octanol: PBS (pH 7.4) using flask shaking method. The values of partition coefficient of natamycin indicated its

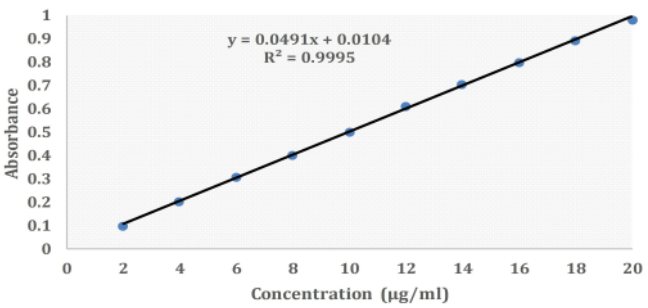

Figure 3. Linearly regressed standard curve of Natamycin in Glacial acetic acid: Methanol (1:1000)

Table 1. Solubility profile of Natamycin in different solvent system

Solvent	Solubility
Distilled Water	Insoluble
Methanol	Sparingly soluble
95% Ethanol	Insoluble
Acetic acid	Soluble
Acetone	Insoluble
PBS (pH 7.4)	Insoluble
Acidic buffer (pH 3.6)	Insoluble
Basic buffer (pH 7.2)	Insoluble
Glacial acetic acid: Methanol (1:1000)	Freely Soluble
Chloroform: Methanol (3:2)	Very soluble

lipophilic nature as it was found to be 1.21 in chloroform: PBS (pH 7.4) and 1.15 in n-Octanol: PBS (pH 7.4). The partition coefficient in both systems is depicted in table 2.

The partition coefficient was higher in case of chloroform: PBS system due to stronger acidic nature of chloroform, thereby resulting in higher partitioning of drug into the chloroform layer compared to that of octanol layer.

Table 2. Partition Coefficient values of natamycin

Medium	Partition coefficient
Chloroform: PBS (pH 7.4)	1.21
n-octanol: PBS (pH 7.4)	1.15

FT-IR analysis

FTIR spectrum showed characteristic peaks and bands which are representing the presence of functional groups which help in the identification of drug. FTIR spectrum of NAT was showing characteristics peaks at 3566, 3492, 3017, 2945, 2317, 1716 and 1638cm^{-1} which are indicating, O-H stretching, N-H stretching (1° amine), =CH stretching, C-H_2 stretching, O-H stretching (Carboxylic group), C=O stretching (1°Carboxylic acid) and C=O stretching (lactone group), respectively (Figure 4). These bending and stretching in FTIR spectrum expressed the structure of NAT and presence of functional groups in the drug moiety which would be responsible for the characteristics of the drug and it would also act as a helping tool for the identification of NAT.

Drug excipients compatibility

DSC curve of NAT depicted a sharp endothermic peak at 218.3ºC (Figure 5A). The DSC curve of the physical mixture (span 60, cholesterol and NAT) depicted endothermic peaks at, 56.6C, 136.4C and 218.5C corresponding to span 60, cholesterol, and NAT respectively (Figure 5B). It showed no significant variations in the endotherms of the individual components. DSC thermograms proved the structural stability of the drug and the drug is compatible with the excipients employed. It can be concluded that the drug samples were authentic and results of the studies were in accordance with

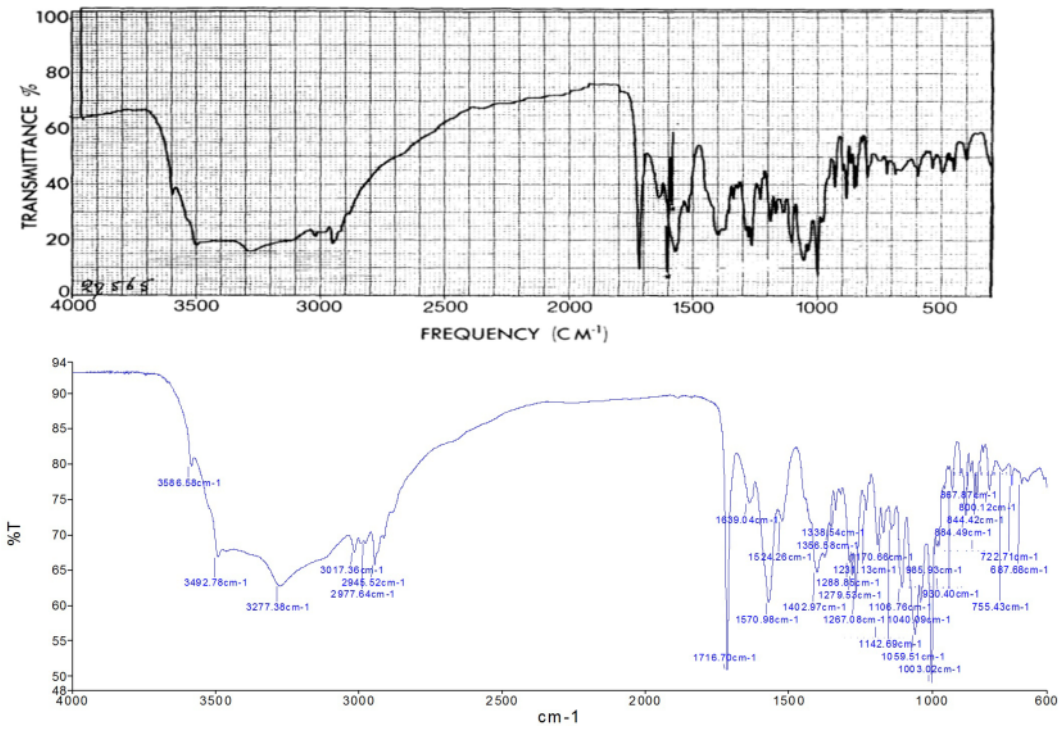

Figure 4. FTIR spectrum of natamycin (a) Standard (b) Observed

the standards. Various analytical techniques confirmed the chemical identity of the supplied drug. The various data generated regarding the partition coefficient, solubility, spectroscopy, standard curves and DSC curves were reproducible and may be used reliably for further work.

Preparation and characterization of NAT loaded niosomes

NAT containing niosomes were prepared by thin film hydration method using cholesterol (34 mg),span 60 (66 mg) and DCP (5% w/w) with 78.61±3.09 percentage drug entrapment efficiency (Jain et al., 2019; Jain and Jain, 2018; Jain et al., 2018). The vesicle size and zeta potential of formulated niosomes were found to be 968±8.24 nm and -22.81±2.19 mV, respectively (Table 3). The drug release study was also conducted for 12 hrs using dialysis bag method and the % drug release was found to be 87.66±4.32 % in 12 hrs (Figure 6) (Jain and Jain, 2016a).

Conclusion

NAT is an effective, broad spectrum antifungal agent and currently employed in different diseases especially for ocular infection. All the studies conclude that the determination of melting point, partition coefficient and FTIR spectral analysis are the excellent tools for the identification of purity of the drug. The drug solubility determination in various polar and non-polar solvents gives an idea about the selection of proper solvent for the preparation of niosomes. The DSC studies of pure drug and with the excipients are showing results regarding drug-excipients interaction where it is concluded that drug is not interacting with the excipients. The appropriate vesicle size, % EE and high zeta potential may render the niosomes as a potential tool in the delivery of natamycin.

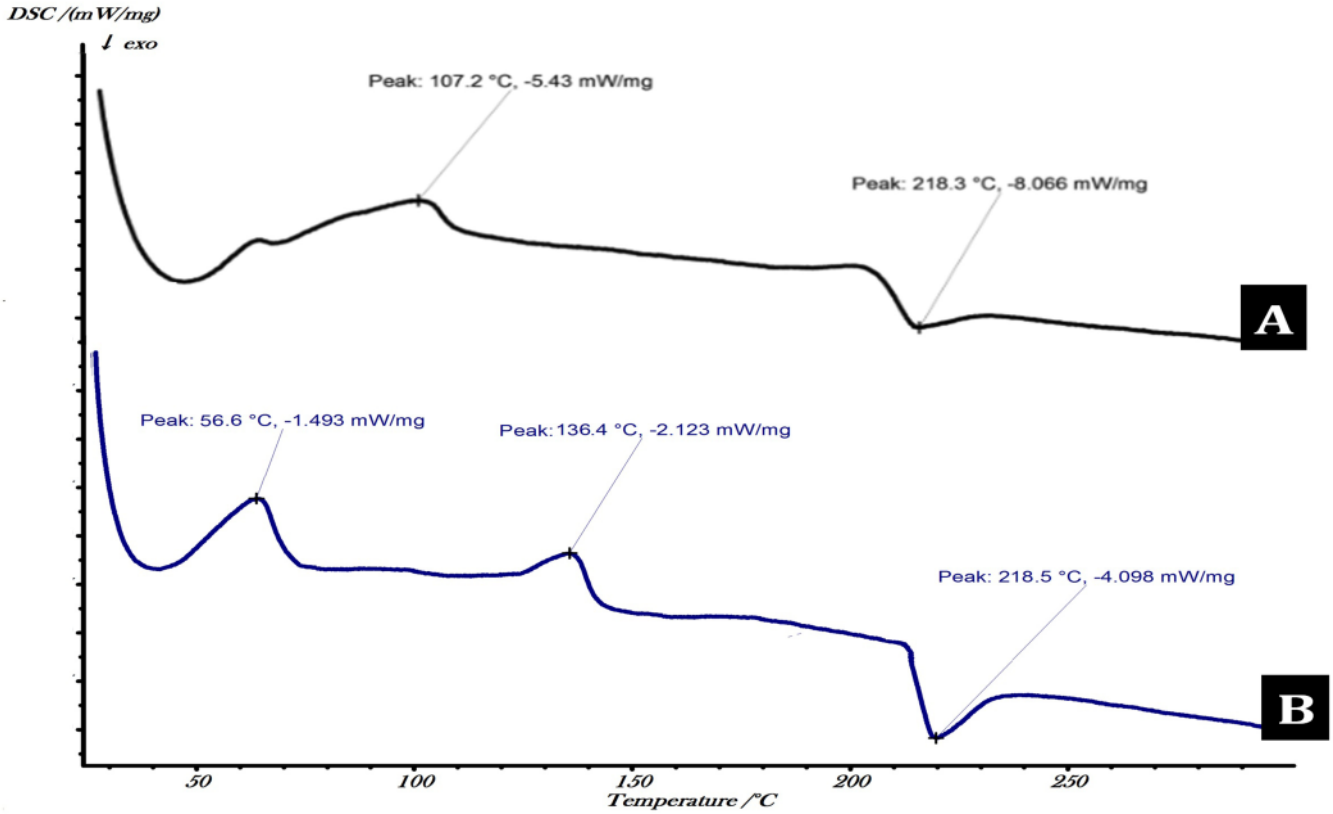

Figure 5. DSC thermogram of (A) natamycin and (B) physical mixture

Table 3. Characterization of niosomes

Parameter s	Value
Vesicle size	968±8.24 nm
Zeta Potential	-22.81±2.19 mV
% EE	78.61±3.09 %

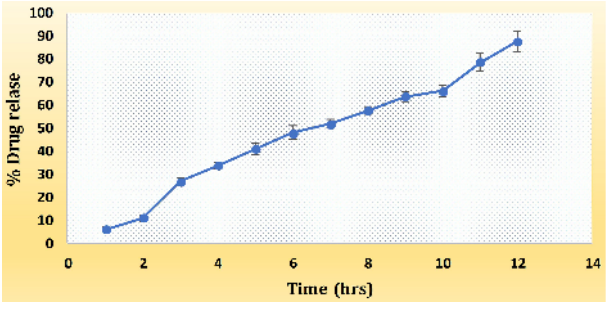

Figure 6. Drug release from natamycin loaded niosomes

Conflicts of interest

The authors declare that they have no conflict of interest.

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