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DEVELOPMENT AND EVALUATION OF CHITOSAN NANOPARTICLES FOR DELIVERY OF ANTICANCER DRUG

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Abstract

Nanocolloidal drug delivery systems are emerging as newer type of delivery system that facilitate the product line extensions through improved therapeutic regimes for existing drug molecules. For the preparation of nanoparticles, water soluble polymers are available and chitosan is one of the most extensively studied. This is because chitosan possesses some ideal properties of polymeric carriers for nanoparticles such as biocompatible, biodegradable, nontoxic and inexpensive. Chitosan nanoparticles were prepared based on the ionic gelation of chitosan with Tripolyphopshate anions. Chitosan was dissolved in acetic acid solution at various concentrations (1.0, 1.2, 1.44, 1.6, 2.0, 2.5, 3.0 mg/mL). The concentration of acetic acid in aqueous solution was in all case, 1.5 times that of chitosan. Under magnetic stirring at room temperature 4ml sodium tripolyphosphate TPP aqueous solution with various concentrations (0.2, 0.4, 0.6, 0.8, 1.0 mg/mL) was added into 10ml chitosan solution, respectively. PEG-modified nanoparticles were formed spontaneously upon incorporation of 4ml TPP solution (0.6 mg/mL) into 10mL chitosan solution containing various concentrations of PEG (10.0, 20.0, 30.0, 40.0, 50.0 mg/mL). The daunorubicin was dissolved in heated distilled water. Daunorubicin loaded nanoparticles were formed upon incorporation of 4ml TPP solution (0.6, 1.0 mg/mL) into 10mL chitosan solutions containing daunorubicin (0.1, 0.2, 0.3, 0.4, 0.5 mg/mL). The formulated nanoparticles were then evaluated for various physicochemical parameters of the dosage form.

Key Words; Tripolyphosphate, Chitosan, Daunorubicin, Nanoparticles

Introduction

Cancer has a physiological barrier like vascular endothelial pores, heterogeneous blood supply, heterogeneous architecture etc. The treatment of cancer depends on the delivery method. The cancer patients are using various anticancer drugs but these are less effective and have major side effects. Drug carriers can be engineered to slowly degrade, react to stimuli and be site-specific. The ultimate aim is to control degradation of drug and loss, prevention of harmful side effects and increase the availability of the drug at the disease site [1] Different carrier systems aims to control the drug release and improve the efficacy and selectivity of formulations. The controlled drug release systems provide appropriate response at the required site of action for prolonged time periods improving the treatment. These systems can be administered by different routes including

intravenous, ocular, oral, intra peritoneal, intramuscular, subcutaneous and cutaneous [2] Due to their multifunctional character, nanoparticles have great interest of scientist. The drug delivery nanoparticles are the latest achievement in the treatment of cancer [1] Nanoparticles have greater surface area to volume ratio, that helps in diffusion process [3] Nanoparticles also leading to special properties such as increased heat and chemical resistance. A single cancerous cell, giving a strain on the nutrient supply and removal of metabolic waste products [4] If small tumor has formed, the normal tissue will not be able to oppose the cancer cells for the normal supply of nutrients from the blood. Nanoparticles are solid colloidal particles with diameters ranging from 1- 1000nm.They consist of drug carriers in which the active ingredient is dissolved, dispersed, entrapped, encapsulated, adsorbed or chemically attached [5]

Chemotherapy with Nanoparticles

Nanoparticles play a very important role in cancer research. Due to extremely small size of nanoparticles they are easily and more readily taken up by the human body. Biological membranes and access cells, tissues and organs are eligible for entrance of nanoparticles. These cells are not able to cross by the larger-sized particles. Nanoparticles are stable, solid colloidal particles consists of biodegradable polymers or lipids and size range 10-1000 nm. Nanoparticles have greater surface area to volume ratio that helps in diffusion process. Nanoparticles also leading to special properties such as increased heat and chemical resistance. A single cancerous cell, giving a strain on the nutrient supply and removal of metabolic waste products. If small tumor has formed, the normal tissue will not be able to oppose the cancer cells for the normal supply of nutrients from the blood stream [6].
The following are among the important technological advantages of nanoparticles as drug carriers: high stability (i.e., long shelf life); high carrier capacity (i.e., many drug molecules can be incorporated in the particle matrix); feasibility of incorporation of both hydrophilic and hydrophobic substances and feasibility of variable routes of administration, including oral administration and inhalation. These carriers can also be designed to enable controlled (sustained) drug release from the matrix [7]

Chitosan is an interesting natural material occurring in abundance in the environment. Its excellent biocompatibility and several advantages due to its unique polymer cationic character renders it highly useful for pharmaceutical application [6] Chitosan possess some ideal properties of polymeric carriers for nanoparticles such as biocompatible, biodegradable, nontoxic and inexpensive. Its positive charge exhibits absorption enhancing effect. These properties render chitosan a very attractive material as a drug delivery carrier. [7] Besides other applications chitosan has been extensively examined for its potential in the development of controlled release drug delivery systems.
Therefore, the major goal of the present study is to create a kind of new biodegradable nanoparticle as drug delivery system. The nanoparticles have been characterized in terms of size, poly dispersity index, and association efficiency. Also in vitro release was investigated to determine the efficacy of the system.

Materials and Methods

Anticancer drug Daunorubicin was received as a gift sample from VHB Life Sciences, Rudrapur, Uttrakhand, India. Glacial acetic acids, tripolyphosphate, potassium dihydrogen phosphate, di-sodium hydrogen phosphate, chitosan, n-ocatanol, PEG 2000 were purchased from S.D. Fine chemicals Ltd., Mumbai (India). Dialysis membrane and sodium lauryl sulfate were from Hi-media Ltd., Mumbai (India). All other reagents used in the study were of analytical grade.

Preparation of Chitosan nanoparticles and Daunorubicin loaded nanoparticles

Chitosan nanoparticles were prepared according to the procedure first reported by Calvo et al., based on the ionic gelation of chitosan with tripolyphopshate anions [8]. Chitosan was dissolved in acetic acid aqueous solution at various concentrations (1.0, 1.2, 1.44, 1.6, 2.0, 2.5, 3.0 mg/mL). The concentration of acetic acid in aqueous solution was in all case 1.5 times that of chitosan. Under magnetic stirring at room temperature 4mL sodium tripolyphosphate (TPP) aqueous solution with various concentrations (0.2, 0.4, 0.6, 0.8, 1.0 mg/mL) was added into 10mL chitosan solution respectively. Three kinds of phenomena were observed: solution, aggregates and opalescent suspension. The zone of opalescent suspension was further examined as nanoparticles. PEG-modified nanoparticles were formed spontaneously upon incorporation of 4mL TPP solution (0.6 mg/mL) into 10mL chitosan solution containing various concentrations of PEG (10.0, 20.0, 30.0, 40.0, 50.0 mg/mL).

The daunorubicin loaded chitosan nanoparticles were prepared in the same manner as plain nanoparticles as reported by Calvo et al., based on the ionic gelation of chitosan with Tripolyphopshate anions [8]. The daunorubicin was dissolved in heated distilled water. Daunorubicin loaded nanoparticles were formed upon incorporation of 4ml TPP solution (0.6, 1.0 mg/mL) into 10ml chitosan solutions containing daunorubicin (0.1, 0.2, 0.3, 0.4, 0.5 mg/mL). Daunorubicin concentration was 0.4 mg/ml for the preparation of PEG-modified nanoparticles loading daunorubicin.

Physicochemical characterization of Chitosan Nanoparticles

The size and zeta potential of the nanoparticles were analyzed by dynamic light scattering in triplicate using a Zetasizer ZS (Malvern Instruments, U.K.) at a detector angle of 90 degree, 670 nm, and 25.0°C. The nanosuspension of daunorubicin was prepared with triple distilled water for size measurement.

Scanning electron microscopy (SEM) was performed using a FEI Quanta 200 SEM FEG operating between 5 and 20 kV. Nanoparticles of daunorubicin were deposited on a thin aluminum plate and dried at room temperature and directly placed on the stub without staining and focused at different magnifications (10000 to 100000 X).

Particle morphology was examined by transmission electron microscopy (TEM) (Moragagni 268D TEM, Netherlands). Samples were immobilized on copper grids. The chitosan nanoparticle suspension with a concentration of 100 ?g/ml was applied drop wise onto a 200-mesh copper grid coated with carbon. After air drying samples were stained with 5 wt% uranyl acetate solution. TEM observation was carried out at an accelerating voltage of 200 kV.

Encapsulation Efficiency and % Recovery

The encapsulation efficiency of nanoparticles was determined by the separation of nanoparticles from the aqueous medium containing non-associated daunorubicin ultracentrifugation. The fabricated nanoparticles formulations were subjected to centrifugation on a cooling centrifuge at 18000 rpm at 4 °C for 50 min. The amount of daunorubicin encapsulated was calculated as the difference between the total amount used to prepare the nanoparticles and amount present in the supernatant. The concentration of daunorubicin was determined spectrophotometrically at 260nm. The daunorubicin percentage encapsulation efficiency (%EE) and percentage recovery (%R) of the nanoparticles were determined in triplicate and calculated as follows:

Encapsulation Efficiency (%EE) = [(total amount of Daunorubicin-free Daunorubicin)/total Daunorubicin] X 100

Recovery (%R) = (Total weight of nanoparticles/ Total weight of polymer, drug and other excipients) X 100

Percent Loading Capacity

Freeze dried chitosan nanoparticles were used for determining drug loading capacity. Daunorubicin was extracted from drug loaded nanoparticles (5 mg) with methanol (20 ml) stirred at 500 rpm at room temperature and dispersion was ultrasonicated (60 magnitude, 0.8 cycles) for 3 min and filtered by 0.2mm membrane filter. The filtered solution was analysed for the content of daunorubicin by spectrophotometric determination at 260nm and drug concentration was calculated by calibration curve. The daunorubicin percentage loading capacity (%LC) of the nanoparticles were determined in triplicate and calculated as follows:

Drug Loading Capacity (%LC) = [(total amount of Daunorubicin /total weight of NPs) X 100

***In- vitro* drug release study**

In-vitro release studies of daunorubicin loaded nanoparticles were performed by dialysis membrane (Hi media Ltd., India) with a molecular weight cut-off of 12000—14000. The membrane opening was tied to the opening of a polyvinyl test tube (1 cm diameter) and dipped in a 100 ml beaker containing phosphate buffer (pH 2.4, 50 ml). The entire system was placed in larger beaker (250 ml) containing distilled water used as outer jacket to maintain the temperature of medium at 37±0.5 °C. A small magnetic bead (Sigma-Aldrich, U.S.A.) was placed in the beaker and was stirred at 100 rpm on a magnetic stirrer (Rolex Scientific, Ambala). At predetermined periods 500 µl of the medium was removed and the amount of daunorubicin was analyzed spectrophotometrically at 260 nm.

Stability Studies

A study was also carried out to assess the stability of chitosan nanoparticles of the drug (Drug-to-polymer ratio 1:1). This was carried out according to the procedure described by Zhang et al [9]. For this purpose the samples were taken in borosilicate glass vials and sealed and the vials were stored in room temperature (15° to 20°C), refrigerator (3° to 5°C) and 37°C (relative humidity = 75%) over a period of 3 months. Samples were evaluated at 0, 1, 2 and 3 months for their drug content as well as any changes in their physical appearance and chemical stability.

Statistical Analysis

Data are expressed as the mean ± standard deviation (SD). ANOVA followed by Student's t-test was used for calculating the significance of results (Graphpad, version 2.01, USA). A value of $p < .05$ was considered statistically significant.

Result

Physicochemical characterization of chitosan nanoparticles

Chitosan nanoparticles were formed at an equivalent mass ratio of chitosan to polyanion due to the fact that high or low concentrations of chitosan compared to polyanions tends to decrease the encapsulation efficiency and/or promote aggregation of particles. Unloaded chitosan nanoparticles were measured to have diameters in the range of ~250 nm ~300 nm. The incorporation of daunorubicin caused a slight increase in the mean diameter of chitosan nanoparticles resulting in an approximately 300 nm ~350 nm range in diameter. The results indicate that the mean diameters of chitosan nanoparticles and daunorubicin loaded chitosan nanoparticles were 255.3±0.1 nm and 315.2±0.2 nm respectively, both with narrow size distributions. The increase in the average diameter of

nanoparticles might suggest that daunorubicin molecules were entrapped in the nanoparticles but it could also arise from other effects such as swelling or aggregation of the nanoparticles.

The surface charge of unloaded chitosan nanoparticles ranged from 10.78 ± 1.54 mV. The number of negatively charged groups of the polyanions TPP was responsible for this difference. Positively charged daunorubicin loading did slightly increase the value corresponding to 14.51 ± 2.58 mV. Analysis of particle morphology revealed that chitosan nanoparticles exhibited a well-defined spherical shape with a solid and consistent structure.

Transmission electron microscopy

The structure of daunorubicin-loaded nanoparticles was examined by TEM. Figure 1 shows morphological characteristics of the chitosan nanoparticles. The nanoparticles had an almost spherical shape and were well dispersed without any major aggregation. They showed that the particles have a uniform spherical shape and smooth surface and they are about 255nm in size. Figure 2 shows the Chitosan nanoparticles loaded with the drug i.e. daunorubicin.

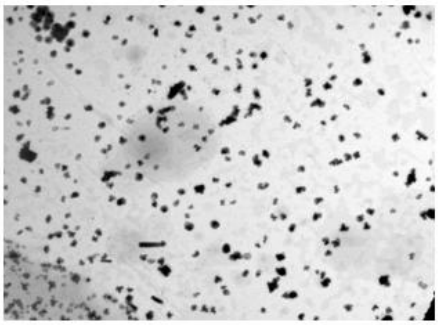

Fig: 1 shows the TEM photomicrograph of Chitosan nanoparticles

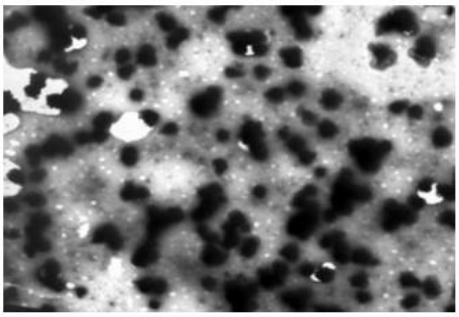

Fig: 2 shows the TEM photomicrograph of Chitosan nanoparticles loaded with daunorubicin

Encapsulation efficiency and % Recovery

In nanoparticle delivery systems the drug carrying capacity is defined as encapsulation efficiency. In the present study daunorubicin was carried on the nanoparticles via the ionic interaction. In water the long hydrophilic chains extended to the water and some drug might be encapsulated among the positive hydrophilic chains which indicated that the daunorubicin was not only on the surface of the nanoparticles but also was distributed in the outer hydrophilic area. So the daunorubicin carrying capacity of the nanoparticles could be termed as encapsulation efficiency. Daunorubicin was successfully entrapped into daunorubicin-chitosan nanoparticles and the nanoparticles had a high EE % of 52.6±0.1% in loading drug. Daunorubicin encapsulation efficiency vary from 40% to 95% and was significantly affected by the initial drug concentration encapsulation efficiency. However the drug loading was enhanced dramatically from 5% to 30% by increasing the initial daunorubicin concentration from 0.1 to 2mg/ml. The efficiency of drug incorporation into nanoparticles was generally limited by the large surface area of the latter as well as by the solubility of the drug in water. These two factors accelerate drug loss into the aqueous phase during nanoparticle preparation. Other factors such as size, geometry and nature of the hydrophobic core of the nanoparticles might also limit the drug loading.

Effect of PEG modification

Polyethylene glycol (PEG) coated nanoparticles have been found to be potential in the therapeutic application for controlled release of drugs and drug delivery to specific sites. Few studies have attempted to investigate chitosan nanoparticles coated with PEG. The PEG coated nanoparticles was conceived with the intention of making these nanoparticles more stable in physiological fluids. The increased size and reduced zeta potential of these nanoparticles is a good indication of the incorporation of PEG in the nanoparticle structure. It has been previously reported that the incorporation of PEG in the gel system is through intermolecular hydrogen bonding between the electro-positive amino hydrogen of chitosan and electronegative oxygen atom of PEG thus forming a CS/PEG semi-interpenetrating network. The interaction between the oxygen atom of PEG and amino groups of chitosan is weak and it still has effect on the nanoparticles formation. The nanoparticle structure modified by PEG is looser, thus the size is larger than that of pure chitosan nanoparticles. Consequently it is not surprising that the increase in the concentration of PEG leads to an increase of the size and a decrease of the positive charge of the nanoparticles. Quellec also reported that the introduction of PEG can decrease significantly the positive surface charge of the particles and noticeably improve their biocompatibility[10]. Polyethylene Glycol was added to chitosan solution prior to gelation. Without TPP incorporation, PEG cannot gelate with chitosan but the amine groups of chitosan can be occupied by the oxygen atom of PEG which may compete in their interaction with the amine groups of chitosan. Thus the possibilities of ion interaction between the daunorubicin and chitosan are reduced. The entanglement of PEG chain with chitosan molecule hinders daunorubicin from encapsulating into the nanoparticles.

Effect of chitosan concentration on encapsulation efficiency

When TPP concentration was 1 mg/ml too high chitosan concentration (4 mg/mL) made encapsulation extremely difficult and too low chitosan concentration (0.5 mg/mL) made some aggregates with large diameter form. The formation of nanoparticles is only possible within some moderate concentrations of chitosan and TPP. As for gelation between TPP solution of 1 mg/ml and chitosan solution of 1–3 mg/ml we usually observed that some opalescent suspension was formed which was further examined as nanoparticles. The increase in chitosan concentration led to decrease of encapsulation efficiency of daunorubicin. It has been previously reported that the highly viscous nature of the gelation medium hinders the encapsulation of drug in the study of chitosan microspheres. So it was supposed that relatively lower viscosity of chitosan with lower concentration (such as 1–3 mg/mL) promotes the encapsulation of daunorubicin and gelation between chitosan and TPP.

Percent loading capacity

The drug-loading capacity of a delivery system is important for minimizing the quantity of delivery system used per milliliter of the solvent. The prepared batches showed good drug-loading capacity. The drug loading varied from 56.71% ± 0.35% to 71.37% ± 0.23% depending upon the drug-to-polymer ratio. The increase of daunorubicin concentration led to an enhancement of loading capacity. (Table 1)

Table: 1 Percentage drug loading for Different daunorubicin loaded chitosan nanoparticles

Drug: chitosan Ratio	Percent Loading Value
1:1	56.37 ± 0.23
1:1.5	63.00 ± 0.21
1:2.0	69.27 ± 0.30
1:2.5	70.83 ± 0.41
1:3.0	71.37% ± 0.23

In-vitro drug release study

The release profile of daunorubicin from chitosan nanoparticles was observed in Phosphate buffer pH 2.4 for 24 hrs. and compared with daunorubicin drug solution. The in vitro protein release profiles obtained for each formulation showed three phases compositions: (1) a first initial burst release of 30% due to the drug desorbed from the particles surface (2) a plateau for the following 8h resulting from the only diffusion of the drug dispersed in the chitosan matrix (3) a constant sustained release of the drug resulting from the diffusion of the daunorubicin through the chitosan wall as well as its erosion. The release of daunorubicin from the chitosan nanoparticles was in sustained manner over a period of 24 h (68.15%) with initial burst release (30% in 1 h). In case of daunorubicin solution 99.82% of drug was released within 4 h with burst effect (51.74% in 1 h). The results indicated that release of drug from nanoparticles was diffusion-controlled as indicated by higher $r2$ values (0.992) in the Higuchi model. It was likely that the rapid release was caused by desorption of daunorubicin loosely attached to the surface of chitosan nanoparticles. The burst release of drug is associated with those drug molecules dispersing close to the microsphere surface, which easily diffuse in the initial incubation time. The hypothesis is also suitable for daunorubicin release from nanoparticles. Since the size of daunorubicin molecule is much smaller than that of nanoparticles, daunorubicin molecules diffuse easily through the surface or the pore of nanoparticles in a short time. The relative small size of the daunorubicin molecule would not be interfered by the diffusion process stemming from the core or the pores of nanoparticles. In addition the high affinity and hydrophilic nature of chitosan with phosphate buffer provides space for penetration inside the particles to be able to dissolve the entrapped drug. Therefore, the rapid dissolution process suggests that the release medium penetrates into the particles due to the hydrophilic nature of chitosan and dissolves the entrapped daunorubicin. In addition the nanoparticles with huge specific surface area can adsorb daunorubicin so the first burst release is also possibly due to the part of daunorubicin desorbed from nanoparticle surface. It should be noted that the molecular size of drug and ionic interaction with polyanions would be a major consideration in deciding the rate of drug release. After 8 hours drug was released very slowly suggesting that the nanoparticles might act as a barrier against the release of entrapped daunorubicin. The hydrolysis and the migration of daunorubicin to release media might be strongly restricted by the hydrophobic core of the nanoparticles. This result indicated that the nanoparticles contributed to an extended circulation of daunorubicin and thus an improvement in therapeutic efficacy.

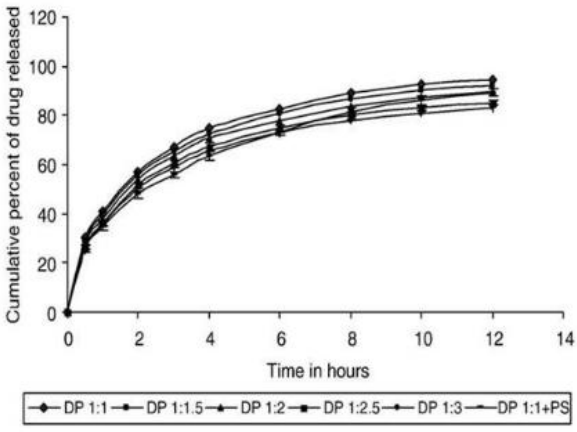

Fig:3 Release kinetics of different chitosan nanoparticles

Release kinetics

The release kinetics was characterized by fitting the data obtained from in vitro–release studies of nanoparticles (drug-to-polymer ratio 1:1) to standard release equations (first-order, Higuchi model and Korsmeyer-Peppas model) [11]. The results obtained are presented in Table 2. The model that best fits the release data is selected based on the correlation coefficient value of various models. The results

indicated that release of drug from nanoparticles were diffusion-controlled as indicated by higher r2 values in the Higuchi model. When the release data were analyzed using the Korsmeyer-Peppas equation, the n values indicated that the mechanism of drug release from the chitosan nanoparticles was Fickian.

Table: 2 Release kinetics of chitosan nanoparticles of the drug daunorubicin for various drug: polymer ratio

Formulation(Drug: polymer ratio)	Release Kinetics			
	First order r2	Higuchi r2	Korsmeyer-Peppas	
			r2	N
1:1	0.1011	0.9422	0.9763	0.2
1:1.5	0.0182	0.9765	0.9941	0.3
1:2.0	0.0180	0.9766	0.9942	0.3
1:2.5	0.0179	0.9769	0.9945	0.3
1:3.0	0.0178	0.9770	0.9951	0.3

Stability Studies

Stability of a drug in a dosage form at different environmental conditions is important because it determines the expiry date of that particular formulation. Changes in the physical appearance, color, odor, taste or texture of the formulation indicate the drug instability. The chemical changes that may occur in the formulations are ascertained through chemical analysis only. Hence the stability and chemical interaction of the drug in the nanoparticles were studied. Table: 3 shows the stability studies results of chitosan nanoparticles (drug-to-polymer ratio 1:1). There were no changes in their physical appearance. The total drug content in the formulations was determined at time 0 and after 1, 2,and 3 months of storage at room temperature (15° to 20°C), refrigerator (3° to 5°C), and 37°C (relative humidity = 75%). It was observed that the initial drug content and the drug contents of the samples analyzed after 1, 2 and 3 months of storage at various conditions were similar indicating there were no significant changes in the physical as well as chemical characteristics of the formulations. Chemical interaction between the drug and polymer, if any during the storage conditions was studied using FT-IR. No significant changes were observed in the IR spectra of the drug-loaded nanoparticles just after the formulation (zero time) and after 3 months of storage. These results indicated that the developed chitosan nanoparticles are physically and chemically stable and retain their pharmaceutical properties at various environmental conditions over a period of 3 months.

Table: 3 Stability studies of chitosan nanoparticles of the drug daunorubicin with a drug-to-polymer ratio of 1:1

Temperature	Evaluation parameter	Observation time in months			
		0 month	1 month	2 month	3month
15–20°C	Physical appearance	Straw yellow	No change	No change	No change
	Drug content (% wt/wt)	13.37 ± 0.23	13.36 ± 0.20	13.36 ± 0.23	13.36 ± 0.21
3-5°C	Physical appearance	Straw yellow	No change	No change	No change
	Drug content (% wt/wt)	13.37 ± 0.23	13.37 ± 0.37	13.37 ± 0.16	13.37 ± 0.22
37°C (RH=75%)	Physical appearance	Straw yellow	No change	No change	No change
	Drug content (% wt/wt)	13.37 ± 0.23	13.37 ± 0.34	13.37 ± 0.16	13.37 ± 0.22

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