RESEARCH PAPER

Development and Evaluation of Enteric Coated Capsules of Freeze Dried Lectin Conjugated Eudragit®S-100 Encapsulated Retinol Acetate Nanoparticles

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ABSTRACT
In these studies, we investigated and evaluated the effect of freeze-drying technology using different cryoprotectants on the resultant products. The results of screening and evaluation of the dried products suggested that the nature of the formulation, as well as the concentration and composition of its components and drying conditions, play an influential role in the drying process. These studies suggested that freeze-dried optimized lectin-conjugated Eudragit®S-100 encapsulated retinol acetate nanoparticles (2:1:1,w/w, Drug (D)/ Eudragit®S-100(E)/Lectin (L)) with 5% w/v mannitol as a cryoprotectant were the most effective. This formulation showed better redispersibility, good flow properties, and comparatively lower particle size (312.2 ±10.0 nm), polydispersity index (0.46±0.09), zeta potential (-10.4±1.1mev), and drug content (82.54 ± 0.62%). The freeze-dried drug/Eudragit®S-100/ lectin nanoparticles with 5% w/v mannitol were incorporated into hard gelatin capsules, which were subsequently enteric-coated. Since the gastrointestinal tract exhibits a wide range of pH conditions, enteric coating was applied to protect the drug and ensure its release in the colon. These capsules were evaluated according to pharmacopeial specifications.

Keywords: *Nanoparticles, Lectin, Conjugation, Shelf-Life, Freeze Drying, Cryoprotectant, Screening, Evaluation, Redispersity, Enteric Coated, Capsule.*
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INTRODUCTION

Lectin conjugated nanoparticles are capable of specific recognition and reversible binding to carbohydrate moieties, glycoconjugates and glycans in body organs[1]. Nevertheless, their full applications have not been exploited due to the lack of stability of formulations when conserved in aqueous medium for a longer period[2]. During storage in colloidal forms, chances of microbiological growth, polymer hydrolysis and physicochemical instability as a consequence of particle aggregation with therapeutic activity loss were occurred[3].

Lyophilisation (freeze drying) is widely used process for pharmaceuticals and biologicals to improve long term stability. It enhances the association of drug product by converting it into solids and removing water from the sample by vacuum desorption or sublimation. During freeze drying cryoprotectants such as sucrose, mannitol and dextrose are added if necessary to protect the sample from stresses for retention of homogeneity. Process of freeze drying involves, freezing where cooling the material until completely frozen. Primary drying involves sublimation of ice from product reducing pressure in the chamber and providing heat to the product. Secondary drying which is desorption of residual moisture from the product[4]. This ensures that nanoparticle aggregates can readily re-disperse into primary nanoparticles in an aqueous environment, thereby retaining their therapeutic functions and particle size. Cryptoprotectants also impart stability during the drying process. Freeze drying is an important processes for drying of thermolabile substances, as it provides the best-controlled process conditions to overcome instability[5]. Therefore, the objective of this study was to evaluate and investigate the efficiency of freeze-drying technologies in transforming ectin-conjugated. Polymer encapsulated drug aqueous nano-dispersions into free-flowing, stable powder with longer shelf life.

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The other objective of the study was to develop and evaluate enteric-coated hard gelatin capsules containing freeze-dried nanoparticle powder. The gastro-intestinal tract has a wide range of pH levels, and enteric-coated products are designed to remain intact in the stomach and release the active drug in the colon[6]. In previous experiments, colon-targeted nanoparticles prepared in our laboratory were tested *in-vivo* through histopathology studies, which showed their potential as colon-specific drug delivery systems. To facilitate the administration of dried nanoparticles, a solid oral dosage form, such as capsule was selected. To improve colon specificity, the chosen hard gelatin capsules, after incorporating the freeze-dried material, were enteric coated with Eudragit®S-100. The purpose was to protect the dried nanoparticles as they pass through the varying environments of the gastrointestinal tract.

Experimental Materials and Methods

Lectin conjugated Eudragit®S-100 encapsulated retinol acetate nanoparticles (2:1:1, w/w, Drug/ Eudragit®S-100 (E) /Lectin). All of these were obtained under optimized parameters using a modified rapid expansion of supercritical solution process with mixing sonication, resulting in a viscous dispersion. Cryoprotectants such as mannitol (Pealitol®SD-200), sucrose **(**D(+)-saccharose, Sugar**),** lactose (Pharmatose® 200M Monohydrate) all were purchased from S.D.Fine Chemicals Ltd., Mumbai India. Agift sample of Eudragit®S-100 from Evonik Germany and empty gelatin capsules of size 00 from Associated Capsules Ltd., India, were used. Isopropanol, acetone, polyethylene glycol (PEG)-6000 and talc were also purchased from S. D. Fine Chemical Ltd., Mumbai. Freeze-dried lectin-conjugated Eudragit®S-100 encapsulated retinol acetate nanoparticle powder with mannitol 5% w/v mannitol as a cryoprotectant was used. These dried nanoparticles were selected based to the best results for drug content percentage and good flow properties. Double distilled water obtained from a Millipore USA system was used throughout the experimentation.

Freeze Drying Procedure for Experimentation

Freeze-drying was performed for Eudragit®S-100 encapsulated retinol acetate nano-suspension (2:1, w/w, Drug / Eudragit®S-100) obtained by the modified RESS process with sonication and mixing at optimized process parameters. The nanoparticles were evaluated and characterized using various tests. Cryoprotectants and adsorbents were selected based on literature reports, and availability and the results of previous experimental trials in the lab[7,8]. Most of the excipients selected were included in the FDA inactive ingredients database for oral, parental routes of application and were GRAS listed.

Freeze drying was performed using the Labanco (ww-3339-97) freeze dryer, USA. Various cryoprotectants such as mannitol, sucrose, and lactose were screened at a concentrations of 1%, 2% and 5% w/v. The screenings of cryoprotectants were conducted based on the redispersibility of the dried nano-dispersion in doubled-distilled water (DDW) and the flow properties of the obtained dried powder. Process time, freezing temperature, pressure were set and monitored through the inbuilt Programmable Logic Controller (PLC), for the various processes. In this process, the samples were first placed in flasks in the freeze-drying chiller assembly. The Initial chilling process was performed to remove water vapors and to freeze the sample through evaporative cooling and freezing. The process was performed by primary and secondary drying at a freezing temperature of -40ºC for 12 hours and -15ºC for 6 hours respectively. Lyophilisation was carried out by applying a vacuum and chilling, allowing the ice to sublimate into water vapour. The process was continued for 20 hours until the complete removal of water from the flask. When the temperature of the flask reached room temperature, external condensation was stopped and the flasks were removed. Samples of the lyophilized material (in the form of powder) were removed from each flask[8].

Evaluation of Freeze Dried Lectin Conjugated Polymeric Drug Nanoparticles

Each resultant lectin conjugated polymeric drug nanoparticles in the dried powder form was studied for various physicochemical *in-process*ed valuations, along with characterization of the dried products. Characterization of the obtained powder was performed using haemagglutination tests, DSC thermoanalysis, and particle surface morphology by SEM. Infrared spectroscopy (FTIR) was carried out to detect the main functional groups in the resultant dried products. Additionally powder flow characteristics were determined. All these tests procedures are described below.

Particle Size and Polydispersity Index (P.I.) Measurement

The particle size distributions were measured by re-dispersing dried sample in double distilled water (10 mg in 10 mL) and analyzing it using dynamic light scattering (ZetaSizer Nano ZS, Malvern Instruments, Worcestershire, U.K.).

Zeta Potential Measurement

The zeta potential was measured by dispersing dried nanoparticles (10 mg in 10 mL) in double-distilled water, filtered through a 0.45 μm membrane filter from Bio-Rad USA, using a Malvern Zetasizer Nano ZS, UK.

Drug Content Determination

Dried nanoparticles (2.5 mg) were dissolved in 10 mL of ethanol/water (50:50 w/w) in 10 mL volume volumetric amber-coloured flask .This solution was further diluted with the same solvent and analyzed spectrophotometrically using a UV-Visible double-beam (V-670, Jasco, Japan) at 326 nm. The drug content (%) was determined by comparison with a standard calibration curve.

Scanning Electron Microscopy (SEM)

The morphological examination of the nanoparticle dispersion (dried nanoparticles, 10 mg in 10 mL DDW) was performed by scanning electron microscopy

(Environmental-SEM).The liquid sample was spread on a double-sided adhesive tape previously adhered to SEM aluminium stubs and then sputter-coated with platinum in an ion sputter for 300 seconds. Images were collected at an acceleration voltage of 15 kV using a backscattered electron detector on a JEOL JSM-6360®SEM, Japan at 25±2°C.

Angle of Repose

The angle of repose was measured by allowing the powder to flow from a funnel fixed to a stand and measuring the radius of the resulting powder heap[16].

It was calculated using the following formula,

$$\tan\theta \left[\frac{\text{Height from thetipof thefunnel to thebase of theheap (h)}}{\text{Radius of thecone(r)}}\right]$$

$$\textbf{Angle of repose } (\theta) = \tan^{-1}(h/r)$$

Compressibility index (C.I.)

Compressibility index is defined as a 100 times the ratio of the difference between the tapped density and the bulk density to the tapped density.

Bulk density is defined as a ratio of weight of powder mass to the volume occupied by the same when it is poured. It was determined by taking 10 g of dried powder in Bulk-Tapped densitometer (Scientific Ltd., India), and measuring its volume. Bulk density was calculated in g/mL using the formula,

Bulk density (ρo) = Mass of powder taken (M) / Apparent unstirred volume (Vo) Tapped density is defined as the ratio of the weight of a powder to the volume occupied by it after sufficient tapping. It was determined by taking 10 g of dried powder in a in a Bulk-Tapped Densitometer (Scientific Ltd., India). Tapped density was calculated in g/mL using the formula:

Tapped density (ρt) = Weight of sample powder taken (M) / Tapped Volume (Vf) From the measured bulk density and tapped density, the compressibility index (Carr's Index) was calculated using the formula,

C.I = Tapped density (ρt) – Bulk density (ρo) / Tapped density (ρt) X 100 Water content (Loss on drying % w/w)

Determined automatically by placing 0.5 g of the sample in the pan of a moisture analyzer

(HB83S) (Meter Toledo, India) for 45 seconds.

Determination of contact angle (º)

The dynamic contact angle between powder compacts and water was measured using the sessile drop method with a Kruss DSA100 instrument and the drop shape was analyzed Kruss DSA1 software (both are from Kruss Ltd., Hamburg, Germany).The static contact angle between 10 µg of sample and water was measured in triplicate.

Fourier Infrared Spectroscopy (FTIR) spectroscopy study

1 mg of dried nanoparticle powder was triturated with 0.1g of potassium bromide (KBr).The pellet was prepared using a KBr press (model-15) from Scientific Ltd., India. The FTIR spectrum was recorded in the range between 4000 and 400 cm^{-1}, at a scan speed of 4mm/s with a resolution of 2 cm^{-1} using FTIR Spectrum RXI spectrometer(Model-LM500, Perkin Elmer Ltd., Germany). A KBr pellet was used as the reference sample.

Differential scanning calorimetric (DSC) study

DSC thermoanalytical measurements were carried out on dried nanoparticles using a Themofischer PVT. Ltd. (DSPT-1000, India) instrument. DSC thermograms were recorded for 5mg of sample under a nitrogen flow of 20 mL/min at a scanning rate of 10°C /min, over a temperature range of 35°C to 310°C. An empty aluminium pan was used as the reference.

Haemagglutination test

Saline, lectin solution and dried lectin-conjugated nanoparticles (100 µl each) were added to a96-well round bottom plate (Bio-Rad Ltd.,India). To each well, 100 µl separated red blood cells from *Albino* rats was added. The plate was then incubated for one hour at room temperature. After incubation, drops of the suspension from each well were placed on a glass slide. A cover slip was applied, and the slides were observed under 1000X magnification using a compound microscope (Eclipse TS100®,Nikon, Japan).

Preparation and evaluation of final dosage form

This stage mainly focused on;

- Development of oral capsule solid dosage form of the dried drug-polymeric lectin conjugated solid micro/nano-particles.
- Enteric coating of capsule dosage form to provide adequate protection in the gastric pH and prevent non-specific binding of the lectin-grafted micro/nano-particles at other potential sites.
- Evaluation of the dosage form, including stability studies.

Preparation of oral enteric-coated capsule solid dosage form of dried drug polymeric lectin-conjugated micro/nano-particles.

Capsule filling was performed manually. Cap and body of empty hard gelatin capsules (size 00) were separated manually. Accurately weighed quantity of freeze dried lectin-conjugated Eudragit® S-100 encapsulated retinol acetate nanoparticles powder was filled manually in the body of the capsule shell. The cap was then locked on the capsule body and pressed manually. The weights of the empty capsule shell and the filled capsule shell were determined. The enteric coating solution was prepared according to the composition listed in the table 1. The capsules were enteric coated using a manual dip coating

and air-drying process. The dip coating and drying-procedure was repeated untilla 10% w/w weight gain was achieved for each filled capsule, using an electronic balance (AY-120, Shimadzu Ltd., Japan) to monitor the weight.

Table 1: Composition of the enteric coating solution

Excipient	Composition w/w (%)	Role
Eudragit®S-100	10.5	Film former
Purified water	2	Vehicle
Isopropanol	41	Vehicle
Acetone	41	Vehicle
PEG-6000	5.0	Plasticizer
Talc	0 .5	*Anti-tacking* agent Avoids sticking

The integrity of the coat applied over the capsule shell was observed. Since the quantity of dried nanoparticles at the laboratory scale was small, a simple manual dip-coating method was adopted.

Similar enteric coating methods have also been reported in previous studies[9, 10].

Evaluation of enteric capsule of retinol acetate nanoparticles

The prepared dosage form was evaluated for physicochemical parameters. The tests recommended in pharmacopoeias for enteric-coated dosage forms were performed as follows: **Appearance**

The capsules were visually inspected for overall appearance and any physical defects.

Weight variation

Weight variation was determined by manually weighing 20 capsules using an electronic balance (AY-120, Shimadzu Ltd., Japan). The average weight and weight variation was calculated.

Gastro-resistance testing (Disintegration test)

The delayed-release, enteric-coated capsules were tested using a USP-compliant disintegration test apparatus (TD-20, Pharma-Chem., Machineries Ltd., Mumbai). Capsules were placed in acid media (0.1N HCl or acetate buffer, pH 4.5) for 2 hours, with discs used to keep them immersed. The capsules were observed for coat integrity and shell stability. Disintegration testing was then continued in phosphate buffer solution, pH7.4[9].

Assay

The drug content was determined using an assay method. The assay was performed, by dissolving the capsule contents in a 50:50 (v/v) ethanol /water system. The drug content (%) was measured at 326 nm using the UV-Visible method and was further confirmed by RP-HPLC.

Dissolution test

For enteric-coated capsules, the dissolution test was performed using a USP-2 apparatus (AT-7, Sotex, USA) with a paddle at 100 rpm with 900 mL of phosphate buffer (pH 7.4), maintained at 37°C. Sampling were taken every hour for up to 9 hours, untill complete drug release was observed. The cumulative drug release over time was determined. A standard method developed for retinol acetate at 326 nm using UV-Visible spectroscopy. Kinetic modelling was also performed[10].

Stability studies of enteric capsule of retinal acetate nanoparticles

Enteric-coated capsules containing freeze dried lectin-conjugated nanoparticle powder were appropriately packed in Al-Al pack (aluminium-aluminium pouch). These were stored at 25ºC/60% RH (long term storage conditions) and 40ºC/75% RH (accelerated conditions) in a temperature and humidity controlled stability chamber (J.P Ltd., India). The stability of the enteric capsules was evaluated by observing appearance, weight variations, disintegration, drug content, assay using RP HPLC (Model- PU2080, Agilent Ltd., Germany) and a double beam spectrophotometer (Model-V-670, Jasco, Japan)[10].

Results and discussion

Experiments were carried out to screen cryoprotectants using the optimized freeze-drying method for all lectin-conjugated polymer-encapsulated drug nanoparticles. The dried products obtained were characterized and evaluated using the above mentioned tests.

Freeze drying

Screening of suitable cryoprotectant system under optimized freeze-drying conditions for all nanoparticle systems using a freeze dryer was performed. The freeze-dried nanoparticle systems were tested for redispersity by dispersing in double-distilled water and observing their flow properties. Mannitol (5% w/v) was found to be the effective cryoprotectant, showing good redispersity and free flowing properties for all three dried nanoparticulate systems.

Particle size and polydispersity index (P.I.) of the redispersed dried nanoparticles indicated nanoparticles dried with 5%w/v mannitol exhibited good good redispersibility, lower particle size and a narrow P.I.(Figure 1). Removal of water from nanoparticle dispersions induced entanglement of polymeric chains, leading to irreversible particle aggregation. To prevent reversible aggregation and retain the redispersibility of nanoparticles, dispersants / cryoprotectants such as sucrose, lactose, and mannitol were used. Fast drying has provided better conditions for preventing chain entanglement, resulting in increased particle size. Similar results were obtained for freeze-dried nanoparticle

dispersions with 5%w/v mannitol, showed relatively lower particles size (313.45 ± 11.02 nm) and narrow P.I. (0.462 ± 0.07). Characterization results for particle size and polydispersity index of the different dried products for the each nanodispersion system are shown in figure 1. The optimized

nanoparticles had a particle size 235.0± 5.45 and P.I. 0.568 ± 0.05. During the drying process, different drying conditions led to an increase in particle size with a wider distribution. Mannitol, due to its excellent protective, carrier, and tonicity adjusting properties, as well as its moisture-adsorbing nature, acted as an effective cryoprotectant during lyophilisation. These results are consistent with the findings reported in references[11,12].

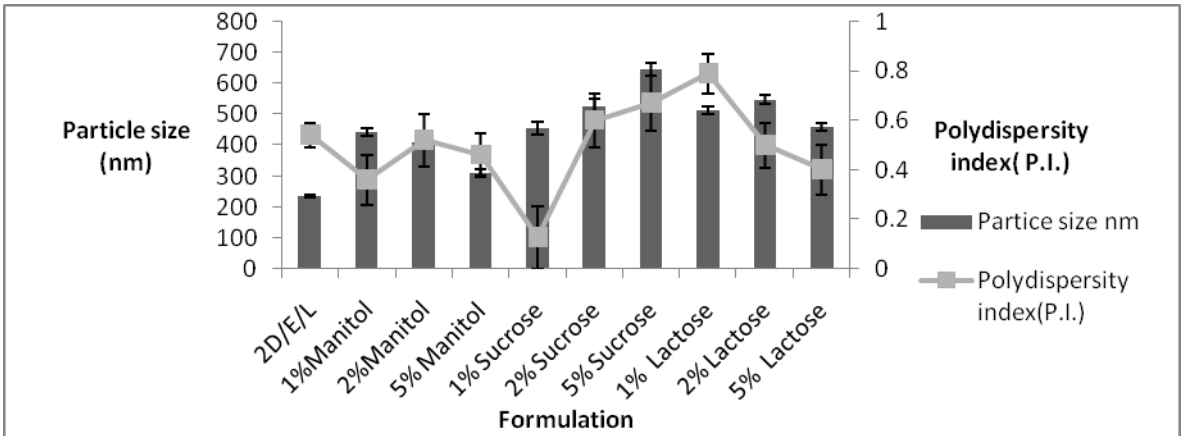

Figure 1: Freeze dried products with optimized lectin-conjugated nanoparticles including particle size and polydispersity index

Zeta-Potential

Comparative data on particle size, polydispersity index (P.I.), zeta potential of lectin-conjugated polymer-encapsulated and optimized dried nanoparticle systems are given in the table 2. It was observed that lectin-conjugated drug encapsulated by Eudragit®S-100 (2D/E/L) nanoparticles, when freeze-dried with 5%w/v mannitol exhibited relatively lower particle size, a narrower polydispersity index, and lower zeta potential, indicating a relatively stable formulation. A decrease in zeta potential was observed in dried products compared to optimized nanoparticles (−15.43 ± 0.43 mV), which may be attributed to the drying process and reduction of the dispersion medium. Thus, the dried product was considered relatively stable. Another possible reason for the change in zeta potential after drying is the alteration of the solvating shell at the nanoparticle surface and interactions with cryoprotectants or adsorbents. Lectin itself also acted as a stabilizer during formulation processing and drying. This confirms the stabilizing effect of cryoprotectants / adsorbents during drying. Similar results were reported in studies[14].

Table 2: Particle size, P.I., zeta potential and drug content of dried nanoparticles

Formulation of Nanoparticles	Particle Size (nm) ± SD	Polydispersity Index(P.I.) ± SD	Zeta Potential(mev) ± SD	Drug Content (%) ± SD
Freeze-dried lectin-conjugated Eudragit®S-100 encapsulated drug with 5% w/v mannitol	313.45 ± 11.02	0.462 ± 0.07	-9.89±1.1	82.54 ± 0.62

Scanning Electron Microscopic (SEM) Imaging

As shown in the Figure 2 all dried nanoparticles were found different, non-uniform shapes. The dispersion of the dried formulation in DDW was found to be viscous, with some particle aggregates, due to different conditions. The sizes of the dried nanoparticles were within the desired range for oral administration, with most nanoparticles falling in the 1000 nm size range for all dried products. During the drying process of the nanodispersions, dissolved polymers precipitated out of solution due to addition of adsorbents/ cryoprotectants, which adsorbed or diffused on the surface of the nanoparticles, resulting in aggregates in the dried products. After freeze-drying, a porous, flattened, fluffy matrix was formed. In the SEM images, the surface morphology of all freeze-dried samples showed porous, uneven, elastic structures with irregular textures, which could facilitate fast reconstitution. These SEM observations were found in good agreement with particle size distribution results of dried products. The optimized all lectin-conjugated nanoparticles appeared bright, fuzzy, and spherical in shape, with particle sizes below 350 nm.

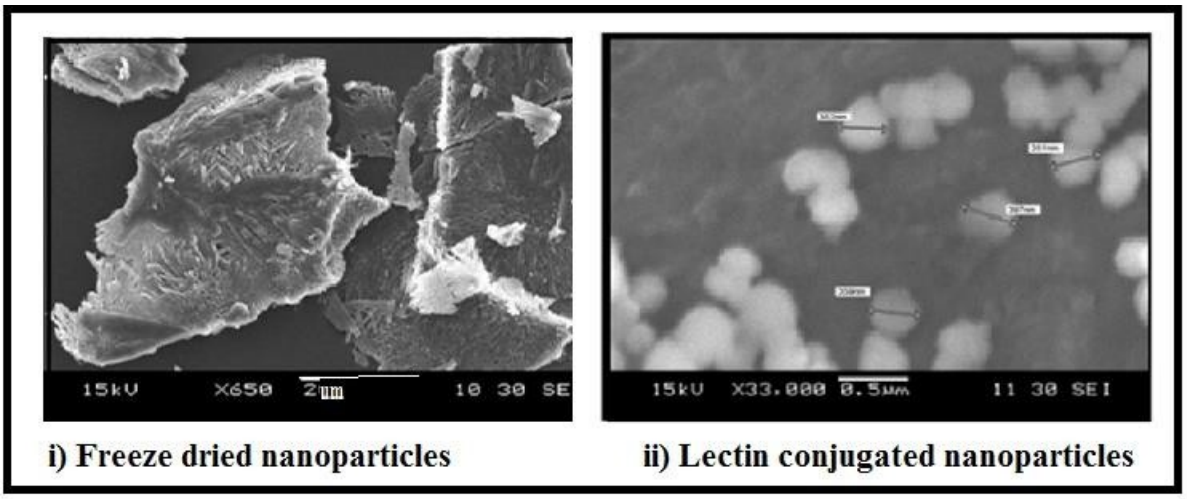

Figure 2: SEM images of dried products with optimized lectin-conjugated (2 Drug / Eudragit®S-100/Lectin) nanoparticles

Due to the drying conditions and the use of components such as cryoprotectants and adsorbents in the processes, the obtained nanoparticles exhibited changes in morphology, with increased size and a broader particle size (P.I.) distribution. These observations are are consistent with the reported findings[13, 14].

Characterization of Dried Products

Flow properties, such as bulk density, tapped density, angle of repose, Hausner's ratio, Carr's Index and drug content (%)were determined using the methods described above to evaluate the efficacy of the drying methods. The results are shown below;

Drug Content (%)

From the observations in the table 2, it was found that drug content (%) was relatively lowered for dried nanoparticles as compared to the optimized lectin-conjugated nanoparticles. This decrease in drug content could be attributed to experimental losses, handling errors and the leaching of drug from the nanoparticles due to the smaller batch sizes. During the drying process, the bursting of nanoparticles led to a loss of drug. Therefore, compared to the initial drug content of 87.84 ± 0.82% for the optimized nanoparticles the drug content in all three dried nanoparticle products obtained by various methods was reduced to 82.54 ± 0.62%.Similar observations were reported previous studies[13,15].

Moisture content (%w/w) and contact angle (º)

From the observations dried lectin-conjugated Eudragit®S-100 encapsulated freeze dried nanoparticles (2D/E/L) with 5%w/v mannitol showed a moisture content 2.1±0.2% w/w and a contact angle 30.2±1.1º.

Carr's index (%) and angle of repose(º)

Freeze-dried nanoparticles (2D/E/L) with mannitol 5% w/v exhibited an angle of repose 22.32±1.1º, and a Carr's index of 21.72±1.1%. An angle of repose (□) < 20º indicates excellent flow properties and Carr's index (%) value 5-15% is considered excellent, as suggested by Remington *et al*[16].

Fourier Transform Infra-Red (FTIR) spectroscopy studies

Optimized and dried nanoparticles have shown selective groups of all excipients and drug used. The FTIR spectra of the dried nanoparticle products shows that various drying methods successfully preserve the structure and composition of compounds. The formation of hydrogen bonding was led to shifts in the wavenumbers for the majority functional groups in the dried products. Mannitol showed a prominent hydroxyl peak at a wavenumber of 3479.27cm^{-1} with an additional peak at 3243.82$cm^{-1}$and an ester (-COO) peak at 1021.8cm^{-1}. Lectin showed characteristic amine group at 1630.18$cm^{-1}$and carboxylic acid group at 2925.91cm^{-1}. Encapsulation, hydrogen bonding formation, adsorption, functional group peak position broadening and a lectin conjugation occurred during various processes as shown in Figure 3.During the drying of nanodispersions, dissolved polymers precipitated out of solution due to the addition of cryoprotectants, which adsorbed onto the surface of nanoparticles. As a result, the positions of the majority of the functional groups shifted in the FTIR spectra of all dried products. The β-ionone ring of retinol acetate was observed at 2918.23 cm^{-1}, with an ether group at 1057.57 cm^{-1}, hydroxylic group at 3320.38 cm^{-1}, and the characteristic amine group of lectin at 1653.57 cm^{-1} in the FTIR spectra of optimized lectin-conjugated nanoparticles (HPMCK-100M) encapsulated with retinol acetate. These group positions were altered in samples subjected to adsorption drying with neusilin®US-2, freeze-drying with 5% w/v mannitol, and vacuum drying with 1.5% w/v lactose.

These FTIR observations suggested the protective role of cryoprotectants during the drying processes through a diffusion mechanism. Nevertheless, drying techniques exerted negligible effects on the chemical profile of the developed nanoparticles, as illustrated by the FTIR spectra and drug content analysis results. Cryoprotectants such as

mannitol, lactose or adsorbent like neusilin®US-2 forms a protective capping layer around the nanoparticles through hydrosoluble matrix formation during the drying process. It is shown in the FTIR spectra all dried products. Similar observations were noted by Wasim *et al*[6]. The formation of a hydrosoluble matrix, in which the particles are embedded may facilitates reconstitution of the dried products in DDW. This addresses the issue of nanoparticle instability during the removal of water molecules in the drying process, as noted by Lee J. et al[17].

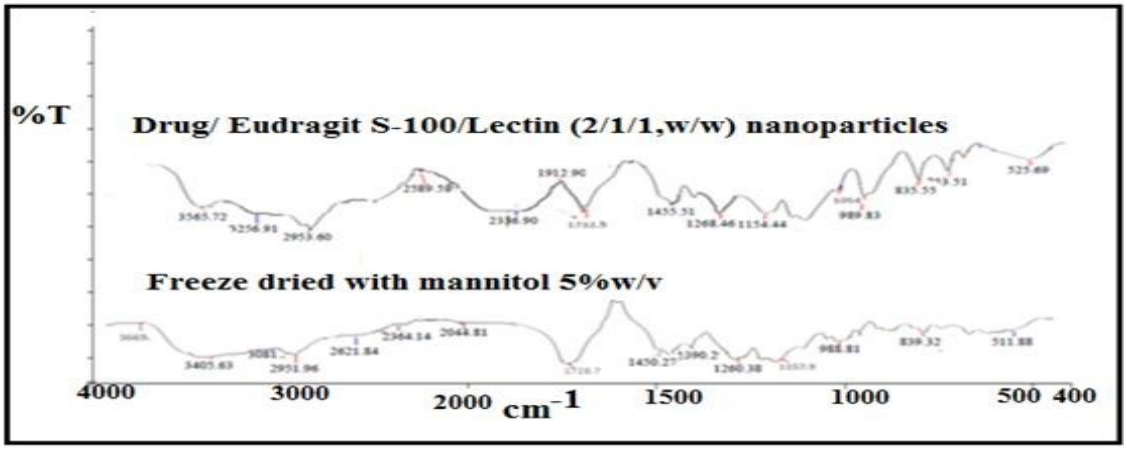

Figure 3.0: IR spectra of optimized and dried nanoparticles (2D/E)

Thus, the drying processes with adsorbents or cryoprotectants were effective in maintaining the structure, composition, integrity and characteristics of the nanoparticles during and after the drying processes.

Differential Scanning Calorimetry (DSC) Studies

The DSC thermogram of mannitol showed a peak around 165°C. The melting points of the respective cryoprotectants or adsorbents were reduced with broader peaks observed in all dried nanoparticle systems. As lectin, due to its protein nature precipitated at lower temperature, it did not show distinct melting point peak. DSC thermoanalysis studies showed that, the diffusion of major drying cryoprotectants with lectin-conjugated polymer encapsulated nanoparticles occurred in the dried product. These DSC thermograms showed slight changes in the melting point due to increased lattice defects resulting from the incorporation of cryoprotectants and changes in processing conditions, as indicated by Wasim *et al*[6]. Hence, all obtained nanoparticle powders were amorphous. For the lectin-conjugated 2D/E/L nanoparticles with Eudragit® S-100, the melting point endotherm was observed around 155–160°C, which was previously seen around 165°C in its pure form. Melting point endothermic peaks of all incorporated components were observed for adsorption, freeze-drying, and vacuum-drying products. Changes in the positions of the functional groups resulted in the lowering, merging, or broadening of endothermic melting point peaks of cryoprotectants, as shown in Figure 4.

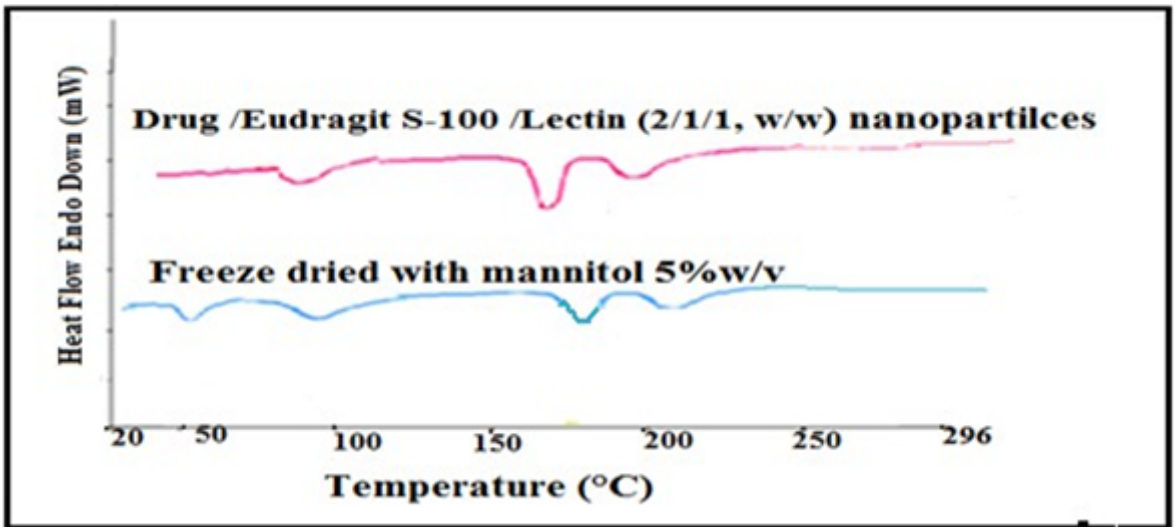

Figure 4: DSC thermograms of optimized and dried nanoparticles (2D/E/L)

No new endothermic or exothermic peaks were observed in the dried nanoparticles indicating that no

incompatibilities between the drug and excipients occurred during the drying process. The total enthalpy change in the dried nanoparticles' melting endotherm was found to be smaller compared to the separate DSC endotherms of each excipient used in the processes. Similar results were obtained by Fernandes *et al*[18].

The main reasons for achieving an amorphous state during SFT processing and drying were highly ordered crystalline structure of the excipients and the processing effects that increased imperfections in the crystal lattice, such as point defects (e.g., vacancies, impurity defects), line defects (e.g., edge dislocations), and plane defects (e.g., grain boundaries). Similar observations were also noted by Brittain H.G. *et al*[19].

Haemagglutination Test

Since lectin is an agglutinating protein and exhibits positive haemagglutination test with RBCs, presence of lectin in the conjugated polymeric nanoparticles after the drying process was confirmed through the haemagglutination test. Agglutination was observed as clots or aggregates of RBCs under the microscope in all dried product samples. The highest intensity of haemagglutination was observed in freeze-dried lectin-conjugated Eudragit® S-100 encapsulated nanoparticles with 5% w/v mannitol, due to the higher percentage of lectin conjugation. The optimized conjugated nanoparticles had 92.68 ± 0.60% lectin conjugations. In contrast, no agglutination was observed in the saline solution. This test confirmed that lectin was preserved during the drying process, as similarly reported by Woodley *et al*[1].

Enteric coated capsules evaluation results

The initial results of various physicochemical parameters of the enteric-coated capsules are summarized below.

Tests		**Results**
Description, appearance	**:**	White to pale yellow, size 00 plain capsule. The capsules were uniformly coated and dry.
Average weight **Average weight**	**:** **:**	Empty hard gelatin capsules : 110 ±4.0 mg Filled capsule : 670.0 ± 6.0 mg
Disintegration test	**:**	
0.1 N HCl	**:**	Capsule shell and coating intact. No evidence of disintegration.
pH 7.4 phosphate buffer	**:**	Average disintegration time of 29 minutes ± 20 seconds.
Dissolution test	**:**	
pH 7.4 phosphate buffer, USP-II, paddle, 900ml.	**:**	100 % drug released in 9 hours.
Assay	**:**	Each capsule average drug content 51.464 ± 2.52mg.

Dissolution Studies:

Sampling for dissolution studies was performed every hour up to 9 hours using the USP-II dissolution apparatus with a paddle. Cumulative drug release over time was determined according to the standard method at a wavelength of 326 nm using UV-Visible spectroscopy. A Graph of the mean cumulative release (%) against time is shown in Figure 5.

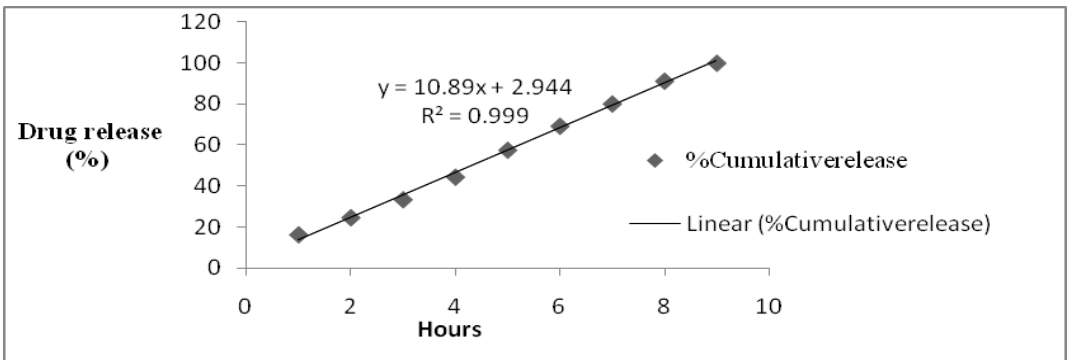

Figure 5: Drug release with time in dissolution studies

The drug release was found to be slow, with a definite quantity of drug being released at the studied time points. A 100 % drug release was observed after 9 hours. This indicates a zero-order release profile and the equation obtained is shown in Figure 5. The Eudragit®S-100 enteric coated capsule containing dried nanoparticles; released the drug by ionization of the free carboxylic group with swelling, as noted by Khan. M. *et al*[20].

Stability Studies

Enteric coated capsules containing freeze dried lectin-conjugated nanoparticle powders were appropriately packed in Al-Al packs (aluminium-aluminium pouch) and monitored over 6 months under two conditions: 25°C/60% relative humidity (RH) (long-term storage conditions) and 40°C/75% RH (accelerated conditions). No significant changes were observed in appearance, average weight, disintegration time, or dissolution drug release over time. The assay of capsule drug content was also monitored at different time points. The drug content of each capsule (%) was found to be within the initial limits. No degradation peaks were detected using the RP-HPLC stability-indicating method, which utilized a mobile phase consisting of acetonitrile and methanol in a ratio of 89:11 (v/v), with a pH of 3.5. The HPLC system consisted of a Plus Intelligent LC Pump® PU-2080 from Agilent, Germany, equipped with an Agilent® UV-2075 Intelligent UV-Visible detector, a Rheodyne® 7725 injector (Rheodyne, Cotati, CA, USA), and Agilent Chromapass Chromatography Data System software (Version 1.8.6.1). The column used was a PurospherStar 5µm Agilent® RP C18 XDB (4.6 mm × 150 mm) at a wavelength of 326 nm.

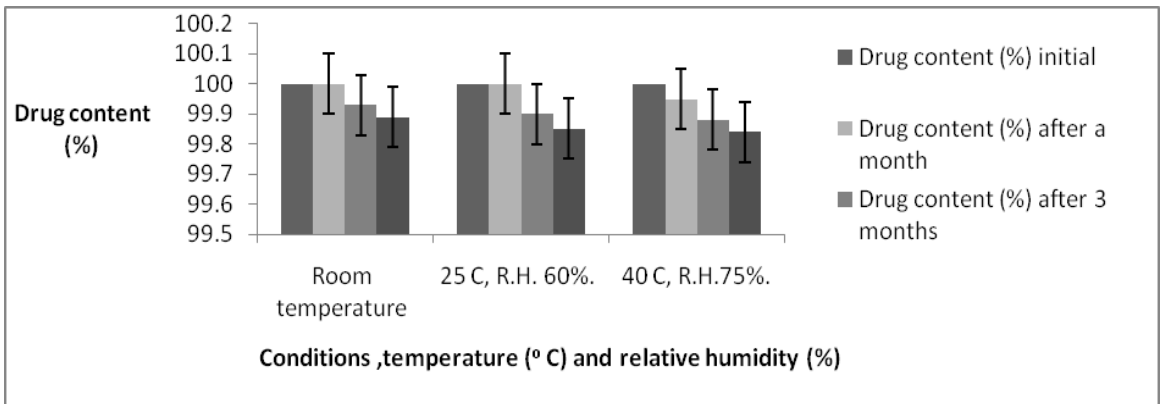

Figure 6: Results of Stability studies

Initially, each capsule's average drug content was found to be 51.464 ± 2.52 mg, which was considered as 100%. Drug content at different time intervals was compared with this value in stability studies. The results are shown in figure 6.

STORAGE

Based on the stability studies, it was observed that for the storage of enteric coated-capsules, a well-sealed, light resistant container or aluminium-aluminium (Alu-Alu) pouch pack is suitable. Further storage of this product in these packs can be done at a temperature of 25°C (77°F), with excursions permitted between to 15° C to 30°C (59°F to 86°F). As an additional precaution, directions to protect the product from light and humidity can be recommended.

CONCLUSIONS

For the lectin-conjugated polymer encapsulated drug nanoparticles, freeze-drying was used to convert them into a stable, therapeutically efficient solid dosage form. When the dried powder was reconstituted in an aqueous system, the redispersed powder achieved its original particle size with only a slight increase. These studies revealed that the drying techniques have considerable effect on the morphological attributes of the nanoparticles as observed in the SEM images of the dried products. All dried nanoparticles were found to be amorphous, as confirmed by their appearance, and DSC thermograms. Nevertheless, the drying techniques showed negligible effects on the chemical profile of developed nanoparticles as illustrated by the FTIR spectra and the drug content analysis results.

Haemagglutination tests, along with FTIR spectroscopy studies have confirmed presence of lectin in all dried nanoparticles. The flow properties of the processed powders were studied as the final dosage form chosen was an oral capsule. These studies demonstrated that for lectin-conjugated Eudragit®S-100 encapsulated retinol acetate nanoparticles (2D/E/L) freeze dried nanoparticles with 5 %w/v mannitol as cryoprotectant was found to be suitable for use in final solid dosage form (capsule). This was confirmed by results of evaluation tests such as particle size, P.I., redispersity in double distilled water (DDW), moisture content, flow properties and drug content, and zeta potential of the dried product.

For nanoparticle dosage forms, wherein the quantity of active drug substance is low, additives are preferred during the drying process. Therefore, screening and evaluation of different adsorbents or cryoprotectants with nanoparticles were successfully conducted. Thus drying of nanoparticles was found to be a complex process

influenced by various parameters such as drying conditions, formulation characteristics, nature and the concentration of adsorbents / cryoprotectants. These studies indicate that the industrially feasible, and cost-effective freeze-drying processes can be suitably employed.

The final objective of the work was to prepare oral colon targeted delivery system was successfully achieved. Hard gelatin capsules were manually filled with the dried nanoparticle powder blend and these capsules were enteric coated manually by using optimized Eudragit®S-100 polymeric coating solution. The prepared capsules were tested for several physicochemical attributes as well as *in-vitro* dissolution testing, which confirmed their suitability as colon-specific drug delivery systems. Enteric coating with Eudragit®S-100 provided adequate protection in an acidic environment. The disintegration of finished product was studied in gastric media 0.1 N hydrochloric acid (HCl) followed by pH 7.4 phosphate buffer. Dissolution drug release studies were also performed in pH 7.4 phosphate buffer. The obtained dissolution data demonstrated that no release occurred in 0.1 N HCl, and continuous prolong release for 9 hours was observed in pH 7.4 phosphate buffer. This drug release was found to be pH- and time-dependent, which can beattributed to the nature of polymer and drug used in the formulations. Thus Eudragit®S-100 has aided in protecting the active ingredient from the gastric fluid and has also improved drug effectiveness by achieving the required GI targeting. *In vitro* drug release was found to follow zero-order kinetics for finished product capsules**.** Thus approach for colonic delivery of lectin- conjugated micro/nano-particles was successfully achieved by enclosing the freeze-dried lectin-conjugated Eudragit®S-100 encapsulated retinol acetate nanoparticle powder with 5%w/v mannitol as cryoprotectant in enteric coated hard gelatin capsules. This formulation prevents non-specific binding of the lectin-grafted micro/nano-particles at other potential sites and ensures targeting to the colon specifically.

Thus, enteric-coated capsule oral dosage form was found to be feasible and can be used as an approach for developing stable colon-targeted drug delivery systems. This dosage form can be explored for other nanoparticles or drug delivery systems targeting the colon in the future.

Declaration

The authors report no conflicts of interest. The authors alone are solely responsible for the content and writing of the paper.

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