ORIGINAL ARTICLE

Comparative Physical and Chemical Stability Studies of Orlistat Liposomal Drug Delivery Systems

Janbandhu Nitesh V, Mogre Ranjan M

Formulation and Development, AVTOS Life Sciences Pvt. Ltd., Navi Mumbai, Maharashtra, India

Abstract

Aims: The aim of the study deals with the design, development, and comparative stability studies of optimized formulations as well as potential formula and process optimization trials of novel orlistat liposomal dispersion. All the experimental studies were evaluated for stability study profiles with respect to physical and chemical key parameters such as physical appearance, pH, density, drug entrapment efficiency, drug content, particle size, polydispersity index and zeta potential. The stability study was carried out at different temperature storage conditions. **Materials and Methods:** Formula optimization studies were utilized to investigate the impact of different molar ratios of orlistat, soya phosphatidylcholine, and cholesterol. Process optimization studies were also performed to comprehend the effect of variable stirring time, sonication time, and centrifugation time on the physical and chemical factors. Orlistat liposomal formulations were prepared using ethanol injection method. **Results and Discussion:** Percentage entrapment efficiency of F30 and F31 has 90.6 ± 0.3% and 89.0 ± 0.2% and average particle size of 847.9 nm and 848.1 nm, respectively, and rest of the physical and chemical parameters were satisfactory. **Conclusions:** The basic aim of the study was achieved with maximum % entrapment efficiency with smaller particle size. The optimized formulations have satisfactory physicochemical parameters at the initial time point and retained similar during stability at long-term storage conditions up to 6 months. Optimized formulations were stable up to 1 month at accelerated storage condition, but there was a significant change observed at 2 and 3 months.

Key words: Liposome, optimization study, optimized formulation, orlistat, stability study

INTRODUCTION

Liposomes are one of the most potent candidates for drug carrier systems. However, the efficacy of liposomes as a drug delivery system has not yet been truly established. One of the reasons for this is the instability of vesicles, particularly in biological media (i.e., blood and gastrointestinal [GI] tract.).[1] In general, in the early stages of development, freshly prepared liposomes are used. However, from a pharmaceutical point of view, it is important to demonstrate that liposomes can be stored for a long period of time. Degradation process particularly oxidation and hydrolysis may change the properties of an aqueous liposome dispersion.[2]

Especially, with liposomal product to see the market, it should be stable during the shelf life (storage or transport). In general, a shelf life of at least 1 year is a minimum pre-requisite criterion for a commercial product. First, leakage of drug from the vesicles may take place into the extra liposomal compartment. Second, there is a possibility of liposomal aggregation and/or fusion, which leads to the formation of larger particles.[3-6] These parameters will alter the *in vivo* fate, affecting therapeutic index of the drug. Hydrolysis of phospholipids is one of the parameters likely to cause the formation of fatty acids and lysophospholipids.[7,8] Although under dehydrated storage, there is least possibility of the formulation to encounter hydrolytic degradation. Another aspect to be considered is liposome oxidation.[9] Oxidation

Address for correspondence:
Janbandhu Nitesh V, AVTOS Life Sciences Pvt. Ltd.,
Plot No.: B-22, MIDC, TTC Industrial Area, Chinchavali Village, P. O. Airoli Node, Navi Mumbai – 400 708, Maharashtra, India. Phone: +91-22-27601688.
Fax: +91-22-27690087.
E-mail: nitesh.janbandhu@avtoslifesciences.com

Received: 22-05-2018
Revised: 08-08-2018
Accepted: 26-08-2018

of phospholipids can be minimized using antioxidants or an inert atmosphere.[10]

The use of herbal remedies carries along problems, such as low solubility and hence limited absorption and bioavailability. Such biologically active compounds are also prone to *in vivo* hydrolysis, oxidation, and photolysis, urging the need for stabilization platforms.[11,12] Technologies such as liquid crystal (LC) systems, polymeric and solid lipid nanoparticles, precursors systems for LC, liposomes, and microemulsions have been reported to overcome such limitations.[13] Liposomes have been extensively studied and are used in the treatment of several diseases. Liposomes improve the therapeutic efficacy by enhancing drug absorption while avoiding or minimizing rapid degradation and side effects, prolonging the biological half-life, and reducing toxicity.[14] Liposomes are colloidal vesicles ranging from few nanometers to several micrometers in diameter with one or several lipid bilayers surrounding an inner aqueous compartment.[15,16] Liposomes are biodegradable, biocompatible, nontoxic, and non-immunogenic. Hydrophilic drugs can be encapsulated in the aqueous core, and lipophilic drugs can be entrapped in the lipid bilayers.[17] Several factors such as aqueous volume, membrane rigidity, surface area, and preparation methods of liposomes have influence on the encapsulation efficiency (EE) of liposomes.[18,19]

Obesity refers to a condition of an abnormal accumulation of body fat mass (body mass index >30 kg/m^2) and is often associated with serious medical conditions, including impaired glucose tolerance, insulin resistance, increased blood pressure, altered lipid levels, and other chronic conditions.[20] Although the etiology of obesity is complicated and not well understood, obesity is likely due to an increase in energy excess that results from caloric intake that exceeds energy expenditure.[21] Orlistat is an anti-obesity agent and acts locally in the GI tract to inhibit lipase, an enzyme that is necessary for the digestion of long-chain triglycerides. Orlistat can also impact cardiovascular risk factors including total and low-density lipoprotein cholesterol, blood pressure, and plasma glucose when accompanied by dietary changes.[22] It is minimally absorbed and helps to reduce body weight by inhibiting absorption of ingested dietary fat by approximately 30%. It can be a useful adjunctive therapy for weight loss and maintenance in obese patients willing to undergo changes in diet and exercise routines.[23]

Orlistat, being highly hydrophobic in nature, is an ideal candidate drug for liposome encapsulation. In the present work, two different preparation techniques for liposomes have been attempted for this challenging endeavor.

In our earlier study, the objective was to formulate the orlistat liposomal colloidal dispersion of various compositions. Formula optimization studies were carried out to see the effect of variation in the active pharmaceutical ingredient (API), lipid and cholesterol concentrations on the physical as well as chemical characteristics of the liposomal formulations. Process optimization studies were also accomplished and evaluated to comprehend the effect of different batch sizes, altered stirring time, ultrasonication time, centrifugation time, etc., on the physical as well as chemical parameters of the liposomal vesicles. We had also studied the impact of alternate method like thin film hydration method on the physical parameters as well as on the % EE and drug content.[24]

In the present study, our goal was to evaluate and characterize the comparative stability study profiles of novel orlistat liposomal formulations and conclude the study. The stability studies were accomplished for formula and process optimization batches as well as optimized batches of the orlistat liposomal dispersion with respect to the physical and chemical parameters. In this investigation, we attempted to develop and evaluate the stability study profiles of the key impact of various formulations and process on the physical appearance, pH, density, drug entrapment efficiency, drug content, particle size, polydispersity index (PDI), and zeta potential. The stability study was carried out at different temperature storage conditions.

MATERIALS AND METHODS

Materials

Orlistat API was received as a gift from Murli Krishna Pharma Pvt. Ltd., Pune. PHOSPHOLIPON 90 G (Soybean phosphatidylcholine) is also received as a gift sample from Lipoid Co., Germany. Cholesterol was purchased from Suvchem Laboratory, Mumbai. All other chemicals and reagents used were of analytical grade and used as such without further purification. Water purified by Milli Q system was used for experiments.

Preparation of orlistat-loaded liposomes by ethanol injection method

Orlistat liposomal formulations with different molar ratios were prepared using the ethanol injection method as it gives better entrapment supported by percentage entrapment efficiency study. Accurately weighed and dissolved phospholipon 90 G, cholesterol, and orlistat (in molar ratios) one after other in minimum quantity of ethanol under stirring in a glass beaker. Purified water was taken in other glass beaker under stirring by magnetic stirrer (Make: Remi Elektrotechnik Ltd., Mumbai, Model: 5 MLH Plus) at 1500 rpm at 36–38°C warming temperature. Then, the above orlistat dissolved lipid solution was added in dropwise manner with the help of syringe to the warm purified water (aqueous phase) in 3 min time under stirring. Continued stirring at 1500 rpm for evaporation of ethanol for about 30 min at 36–38°C dispersion temperature. Turbid/translucent uniform slurry was obtained. The resulting dispersion was ultra-sonicated at power output of 100 with pulser "ON" at 30%

by a probe tip sonicator (Make: Biologics Inc., USA, Model: 300 V/T) for about 5–7 min. The probe tip of the ultrasonic homogenizer was just dipped into the dispersion (care should be taken such that the probe tip does not touch the bottom of the glass beaker during sonication). The ambient temperature was maintained during sonication using ice cubes under the beaker in different containers. Excess heat may be generated during sonication, which may damage the lipids. Uniform liposomal dispersion was obtained after sonication. It was then stored at 2–8°C in refrigerator.

Characterization and evaluation of liposomes

Physical appearance, pH, and density

Clarity of the formulations was inspected under light against white and black background in a well-lit cabinet for appearance and clarity. pH of the formulations was measured using digital pH meter.[25] Density (wt/ml) of the liposomal formulations was determined using pycnometer.

Determination of drug entrapment efficiency (% EE)

Ultracentrifugation method was used to determine the EE. The liposomal suspension was subjected for centrifugation at higher speed for a definite time. Later, the supernatant and sediment (pellet) were collected and analyzed for the drug content.[26]

Orlistat liposomal dispersion (approx. 1.4 ml) taken in Eppendorf and centrifuged in Multifuge 3S-R (Make: Heraeus, Germany) at 14,500 rpm for about 2 h for phase separation. This process was used for the optimized formulation trials. The drug was then estimated using HPLC system (Make: Dionex, USA, Model: Ultimate 3000) from the supernatant and pellet phase after centrifugation process. Methanol was used as a diluent for solubilization of supernatant as well as pellet and then taken the absorbance. The drug entrapment efficiency was calculated using the following formula:

$$\% \text{ Entrapment efficiency} = \frac{\text{Total amount of drug added (\%)} - \text{Free drug content (\%)}}{\text{Total amount of drug added (\%)}} \times 100$$

Or

$$\% \text{ Entrapment efficiency} = \frac{\text{Entrapped drug in vesicles (\%)}}{\text{Total amount of drug added (\%)}} \times 100$$

Drug content

The assay of liposomal formulation was determined by dissolving liposomes in methanol as a diluent. Volume was made up to the mark with methanol in volumetric flask and mixed well to ensure the solubility of drug. This solution was then estimated for drug content using HPLC system with acetonitrile, phosphoric acid, and water (860:0.05:140) as a solvent system at a flow rate of 1.0 ml/min. The 20 µL of sample solution was injected, and the absorption was detected at a wavelength of 195 nm.

Digital microscopy

Digital microscope is a variation of a traditional optical microscope that uses optics and a digital camera to output an image to monitor by means of software running on a computer. Digital microscope used in the study was manufactured by Motic, Hong Kong (Model: BTB24-12A) with built-in digital camera with CMOS and CCD imaging sensors. It has inbuilt LED light source and image analysis software Motic Images Plus 2.0 ML with resolution 3.0 MP.

Zeta potential, mean particle size, and size distribution (PDI)

The PDI value is important in that it shows the size distribution of the liposomes, which can correlate to stability. A PDI value of 1.0 indicates a very broad size distribution or presence of large particles or aggregates, which could sediment. An optimum PDI value is 0.30 or less, signifying that 66.7% of all nanovesicles are the same size.[27] The zeta potential is used to evaluate the dispersional stability of liposomal formulations. It characterizes the particle surface charge and gives an indication about repulsive forces between particles, thus allowing one to predict the stability of dispersions.[28]

Zeta potential, average particle size (in nanometers), and particle size distribution (as PDI) of the liposomal suspension were measured by zeta potential analyzer model Zeta PALS (Brookhaven Instruments Co., NY, USA) at 25°C with a dynamic light scattering method. The sample was placed in a quartz cuvette, and the scattered light was detected at a scattering angle (θ) of 90° for particle size measurement. The vesicle size and zeta potential evaluation of liposomal formulations were performed and described by the volume mean diameter in nanometers and zeta potential in millivolts using software.[29] Each liposomal dispersion sample was diluted with distilled water before analysis until the appropriate concentration of particles was achieved.

Stability studies of liposomal suspension

The particle size, size distribution, change in mean particle size with time, and physical appearance of the liposomal suspension are sensible indicators of the kinetic stability of liposomal suspensions. Drug entrapment efficiency, drug content, zeta potential, pH, and density were also evaluated during stability studies for various formulations of orlistat liposomal suspension. The stability studies were accomplished for potential formula and process optimization batches as well as optimized batches of the orlistat liposomal dispersion with respect to the physical and chemical parameters of the liposomal dispersion.

Comparative stability studies were carried out at accelerated storage conditions (25°C ± 2°C, 60% RH ± 5% RH) for up to 1, 2, and 3 months. Long-term storage conditions (5°C ± 3°C) were carried out for up to 1, 2, 3, and 6 months for optimized formulations. Comparative stability studies were also carried out at long-term storage conditions (5°C ± 3°C) for up to 3 months for potential formula and process optimization batches. 25 ml each of formulation samples were kept in HDPE bottle at sealed condition at above-mentioned time intervals. The samples were withdrawn, examined visually for the evidence of discoloration, and evaluated for its physical and chemical stability. The stability data was compiled and compared with the initial parameters of the liposomal suspension.

RESULTS AND DISCUSSION

In general, the shelf life stability of liposomes is determined by the physical and chemical stabilities (uniformity of size distribution and EE, and minimal degradation of all compounds, respectively). By optimizing the size distribution, pH, and ionic strength, liquid liposome formulations can be made stable. As phospholipids usually form the backbone of the bilayer, their chemical stability is important. Two types of chemical degradation reactions can affect the performance of phospholipid bilayers: Hydrolysis of the ester bonds linking the fatty acids to the glycerol backbone and peroxidation of unsaturated acyl chains (if present). The oxidation and hydrolysis of lipids may lead to the appearance of short-chain lipids, and then, soluble derivatives will be formed in the membrane, resulting in the decrease of the quality of liposome products.[30] Moreover, physical processes such as aggregation/flocculation and fusion/coalescence that affect the shelf life of liposomes can result in loss of liposome-associated drug and changes in size. Aggregation is the formation of larger units of liposomal material; these units are still composed of individual liposomes. In principle, this process is reversible, for example , by applying mild shear forces, by changing the temperature, or by binding metal ions that initially induced aggregation. However, the presence of aggregation can accelerate the process of coalescence of liposomes, which indicates that new colloidal structures are formed.[31] As coalescence is an irreversible process, the original liposomes cannot be retrieved. A colloidal dispersion is often thermodynamically unstable. Spontaneous processes occur in the direction of decreasing Gibbs free energy; therefore, the separation of a two-phase dispersed system to form two distinct layers is a change in the direction of decreasing Gibbs free energy. There is more surface energy in a liposome suspension when the dispersed phase is in a highly subdivided state than when it is in a coarser state of subdivision. The central feature of coalescence is the fact that the total surface area is reduced in the coarsening process of thermodynamically unstable liposome dispersion, while there is no reduction of surface in aggregation, although certain surface sites may be blocked at the points at which the smaller particles touch.[32]

List of formula and batch details of stability batches of liposomal formulations are tabulated in Table 1. The two optimized formulations were kept for the stability studies at accelerated storage conditions (25°C ± 2°C, 60% RH ± 5% RH) up to 3 months and long-term storage conditions (5°C ± 3°C) up to 6 months. The physicochemical parameters of F30 and F31 are presented in Tables 2 and 3, respectively. Physicochemical parameters of potential formula and process optimization batches were also carried out at long-term storage conditions (5°C ± 3°C) for up to 3 months, and the details are given in Tables 4-11.

Based on the stability study data of optimized formulations of B. No. F30 and F31, there were no significant differences observed for physical as well as chemical parameters for long-term storage conditions (5°C ± 3°C) for up to 6 months. Physical appearance, pH, and density of both the batches were retained as similar to initial. The physical appearance of the liposome formulations seemed unchanged, and neither sedimentation nor flocculation was observed indicating the high physical stability of the liposomal system in general. Percentage drug entrapment efficiency, assay, average particle size, PDI, and zeta potential were also satisfactory up to 6 months. However, there were significance differences observed in physicochemical parameters at accelerated storage conditions

Table 1: List of prime formulations and batch details of stability batches of liposomal formulations

Batch No.	Lipid: Cholesterol: API molar ratios	Batch details
Formula optimization trials		
F17	0.62:0.02:0.36	Low lipid and high API concentration
F18	0.86:0.02:0.12	High lipid and low API concentration
F19	0.77:0.01:0.22	Low cholesterol concentration
F20	0.75:0.03:0.22	High cholesterol concentration
Process optimization trials		
F21	0.76:0.02:0.22	Low stirring time (30 min)
F22	0.76:0.02:0.22	High stirring time (60 min)
F23	0.76:0.02:0.22	High sonication time (10 min)
F24	0.76:0.02:0.22	High sonication time (15 min)
Optimized formulation trials		
F30	0.76:0.02:0.22	Optimized stirring time: 30 min Optimized sonication time: 7 min
F31	0.76:0.02:0.22	Optimized stirring time: 30 min Optimized sonication time: 7 min

API: Active pharmaceutical ingredient

Table 2: Stability study data of optimized formulations (Batch No.: F30)

Physical and chemical parameters	Initial	5°C±3°C				25°C±2°C, 60% RH±5% RH		
		1 month	2 months	3 months	6 months	1 month	2 months	3 months
Physical appearance	Milky-white, turbid uniform dispersion	Milky-white, turbid uniform dispersion	Milky-white, turbid uniform dispersion	Milky-white, turbid uniform dispersion	Milky-white, turbid uniform dispersion	Milky-white, turbid uniform dispersion	Lumps, separation and non-uniform consistency observed	Lumps, separation and non-uniform consistency observed
pH	5.18	5.00	5.02	5.42	4.31	3.81	3.77	3.92
Density (g/ml)	1.004	1.050	1.010	1.076	1.070	1.055	1.034	1.075
% Entrapment efficiency	90.6±0.3	89.6±0.6	92.3±0.5	90.6±0.5	95.4±0.4	88.6±0.5	92.3±0.3	90.6±0.5
% Assay	102.0±0.4	102.5±0.7	102.3±0.6	102.1±1.5	101.2±0.7	103.0±2.2	91.3±2.3	96.7±1.1
Average particle size (nm)	847.9	919.5	891.2	1003.8	1122.3	1133.9	1876.9	1571.1
Polydispersity index	0.351	0.338	0.291	0.403	0.410	0.198	0.247	0.957
Zeta potential (mV)	−36.9	−40.4	−41.7	−35.9	−33.6	−32.7	−26.6	−42.1

Data are represented as mean±SD (*n*=3)

Table 3: Stability study data of optimized formulations (Batch No.: F31)

Physical and chemical parameters	Initial	5°C±3°C				25°C±2°C, 60% RH±5% RH		
		1 month	2 months	3 months	6 months	1 month	2 months	3 months
Physical appearance	Milky-white, turbid uniform dispersion	Milky-white, turbid uniform dispersion	Milky-white, turbid uniform dispersion	Milky-white, turbid uniform dispersion	Milky-white, turbid uniform dispersion	Milky-white, turbid uniform dispersion	Lumps, separation and non-uniform consistency observed	Lumps, separation and non-uniform consistency observed
pH	5.07	5.50	5.14	5.41	4.40	3.82	3.70	3.97
Density (g/ml)	1.005	1.044	1.024	1.074	1.065	1.041	1.059	1.075
% Entrapment efficiency	89.0±0.2	89.6±0.8	89.2±0.9	87.3±0.5	95.7±0.5	89.5±0.3	106.0±2.8	96.1±0.2
% Assay	103.8±0.9	102.8±0.5	103.8±1.1	102.9±0.9	100.6±0.6	101.2±0.2	112.9±3.3	114.9±0.3
Average particle size (nm)	848.1	891.2	940.8	920.3	1245.7	1103.3	1508.8	2005.1
Polydispersity index	0.113	0.120	0.210	0.462	0.478	0.489	0.397	0.686
Zeta potential (mV)	−38.8	−41.7	−31.2	−47.5	−39.8	−43.3	−36.9	−29.6

Data are represented as mean ± SD (*n*=3)

Table 4: Stability study data of formula optimization batches (Batch No.: F17)

Physical and chemical parameters	Initial	5°C±3°C	
		1.5 months	3 months
Physical appearance	Some big white particles observed in turbid dispersion. It may be due to higher API concentration	Some big white particles observed in turbid dispersion. It may be due to higher API concentration	Some big white particles observed in turbid dispersion. It may be due to higher API concentration
pH	5.46	5.50	5.68
Density (g/ml)	1.007	1.015	1.020
% Entrapment efficiency	85.9±0.1	85.2±3.6	85.4±4.0
% Assay	97.6±0.6	95.8±0.9	93.8±0.3
Average particle size (nm)	467.1	---	498.8
Polydispersity index	0.552	---	0.375
Zeta potential (mV)	−4.5	---	−7.3

Data are represented as mean±SD (*n*=3). API: Active pharmaceutical ingredient

Table 5: Stability study data of formula optimization batches (Batch No.: F18)

Physical and chemical parameters	Initial	5°C±3°C	
		1.5 months	3 months
Physical appearance	Milky-white, turbid uniform dispersion	Milky-white, turbid uniform dispersion	Milky-white, turbid uniform dispersion
pH	5.35	4.53	3.90
Density (g/ml)	1.002	1.012	1.010
% Entrapment efficiency	62.4±0.1	70.9±0.3	78.4±0.3
% Assay	103.5±0.5	102.3±0.5	104.8±0.7
Average particle size (nm)	569.8	---	864.9
Polydispersity index	0.110	---	0.079
Zeta potential (mV)	−27.5	---	−30.6

Data are represented as mean±SD (*n*=3)

Table 6: Stability study data of formula optimization batches (Batch No.: F19)

Physical and chemical parameters	Initial	5°C±3°C	
		1.5 months	3 months
Physical appearance	Milky-white, turbid uniform dispersion	Milky-white, turbid uniform dispersion	Milky-white, turbid uniform dispersion
pH	6.09	5.39	4.29
Density (g/ml)	1.004	1.020	1.024
% Entrapment efficiency	61.0±1.6	78.4±3.5	80.7±1.6
% Assay	102.6±0.2	104.1±0.9	103.9±1.2
Average particle size (nm)	520.2	---	650.7
Polydispersity index	0.330	---	0.112
Zeta potential (mV)	−10.7	---	−7.2

Data are represented as mean±SD (*n*=3)

(25°C ± 2°C, 60% RH ± 5% RH) for both the batches. Both the batches were stable physically and chemically up to 1 month that accelerated storage conditions and thereafter changes observed in physical consistency and pH up to 3 months. There was no significant impact observed on % EE, but significant variation in assay was observed for both the batches in 2 and 3 months' time point. This may be because of separation and uneven consistency of the dispersion. Particle size of both the

Table 7: Stability study data of formula optimization batches (Batch No.: F20)

Physical and chemical parameters	Initial	5°C±3°C	
		1.5 months	3 months
Physical appearance	**Milky-white, turbid uniform dispersion**	**Some small white particles in turbid dispersion**	**Some small white particles in turbid dispersion**
pH	6.13	5.80	5.04
Density (g/ml)	1.004	1.008	1.010
% Entrapment efficiency	72.8±0.4	77.6±0.3	83.3±0.8
% Assay	101.7±0.3	102.1±0.7	104.5±0.4
Average particle size (nm)	468.2	---	780.9
Polydispersity index	0.560	---	0.491
Zeta potential (mV)	−51.2	---	−42.6

Data are represented as mean±SD (*n*=3)

Table 8: Stability study data of process optimization batches (Batch No.: F21)

Physical and chemical parameters	Initial	5°C±3°C	
		1.5 months	3 months
Physical appearance	**Milky-white, turbid uniform dispersion**	**Milky-white, turbid uniform dispersion**	**Milky-white, turbid uniform dispersion**
pH	6.38	5.63	4.10
Density (g/ml)	1.000	1.020	1.015
% Entrapment efficiency	76.1±0.7	80.9±0.6	83.3±0.5
% Assay	103.6±0.9	103.1±0.4	100.7±1.1
Average particle size (nm)	380.3	---	420.2
Polydispersity index	0.560	---	0.628
Zeta potential (mV)	−26.3	---	−30.6

Data are represented as mean±SD (*n*=3)

Table 9: Stability study data of process optimization batches (Batch No.: F22)

Physical and chemical parameters	Initial	5°C±3°C	
		1.5 months	3 months
Physical appearance	**Milky-white, turbid uniform dispersion**	**Milky-white, turbid uniform dispersion**	**Milky-white, turbid uniform dispersion**
pH	6.30	4.85	3.88
Density (g/ml)	0.992	1.011	1.002
% Entrapment efficiency	78.6±0.2	87.1±0.6	85.1±0.7
% Assay	104.3±0.5	106.3±0.3	102.5±1.4
Average particle size (nm)	386.0	---	433.8
Polydispersity index	0.567	---	0.610
Zeta potential (mV)	−23.6	---	−28.7

Data are represented as mean±SD (*n*=3)

batches was increased significantly in 2 and 3 months' time point. It has been earlier reported that the chemical hydrolysis may lead to an increase in particle size[33] and to an increase in permeability of liposomal bilayers.[34] Zeta potential of both the batches was satisfactory up to 3 months. Digital micrographs [Figures 1 and 2] revealed that the liposomes were spherical in shape and the formed vesicles were in the form of individual particles with sharp boundaries. The photographs were taken for the optimized liposomal formulations. The micrographs showed here attest successful formation of lipid vesicles using ethanol injection method.

Stability study of formula optimization batches like low lipid and high API concentration trial (F17) has some big

Table 10: Stability study data of process optimization batches (Batch No.: F23)

Physical and chemical parameters	Initial	5°C±3°C	
		1.5 months	3 months
Physical appearance	Some big white particles observed in turbid dispersion	Some small white particles observed in turbid dispersion	Some small white particles observed in turbid dispersion
pH	6.24	5.21	4.79
Density (g/ml)	0.990	0.998	1.008
% Entrapment efficiency	72.5±0.8	81.3±0.1	70.5±0.2
% Assay	104.1±0.4	104.3±0.7	105.1±0.5
Average particle size (nm)	560.8	---	598.7
Polydispersity index	0.281	---	0.391
Zeta potential (mV)	–82.4	---	–90.0

Data are represented as mean±SD (*n*=3)

Table 11: Stability study data of process optimization batches (Batch No.: F24)

Physical and chemical parameters	Initial	5°C±3°C	
		1.5 months	3 months
Physical appearance	Some big white particles observed in turbid dispersion	Some small white particles observed in turbid dispersion	Some small white particles observed in turbid dispersion
pH	6.04	6.03	5.20
Density (g/ml)	1.030	1.021	1.018
% Entrapment efficiency	39.4±4.8	73.4±0.2	68.7±0.4
% Assay	110.9±0.4	112.9±0.9	109.1±0.6
Average particle size (nm)	510.2	---	988.4
Polydispersity index	0.311	---	0.525
Zeta potential (mV)	–89.1	---	–80.6

Data are represented as mean±SD (*n*=3)

white particles observed in the turbid dispersion at initial time point. It may be due to the higher API concentration in the suspension. % EE, drug content, and particle size of the suspension were satisfactory. Zeta potential was on lower side. All the parameters remained comparable up to 3 months. High lipid and low API concentration trial (F18) observed uniform dispersion with low % EE, satisfactory particle size, zeta potential, and better PDI at initial time point. % EE was significantly and subsequently increased in 1.5 and 3 months. The particle size of liposomes was also increased in 3 months. This may be due to low API content in the suspension which was further entrapped within the high amount of lipidic core. Low cholesterol content (F19) was observed uniform dispersion with low % EE, satisfactory particle size, PDI, and comparatively low zeta potential at initial time point. % EE was significantly increased in 1.5 and 3 months. Other parameters were comparable up to 3 months. Uniform dispersion obtained in high cholesterol trial (F20) with satisfactory % EE, particle size, and zeta potential but larger PDI at initial. Some small white particles were observed in turbid dispersion and % EE was subsequently increased in 1.5 and 3 months.

Process optimization trials such as high stirring time (F21 and F22) had observed uniform dispersion with satisfactory % EE, drug content, average particle size, and zeta potential but larger PDI. Similar parameters were retained up to 3 months. High sonication trials (F23 and F24) had observed some big white particles in turbid dispersion at the initial time point, and later, also some small white particles were observed in 1.5 and 3 months. F23 was seen with satisfactory % EE, particle size, PDI, and zeta potential at initial, and further, it was retained up to 3 months. F24 was found low % EE and high drug content but satisfactory particle size, PDI, and zeta potential at initial and consequently increased % EE, particle size, and high drug content in 1.5 and 3 months. Therefore, it can be concluded that the very high ultra-sonication time was not suitable for this drug. The vesicles may get disrupted and drug is not entrapped considerably into the lipids.

CONCLUSION

Orlistat API is highly unstable molecule at room temperature, and hence, the storage is recommended in the refrigerator.

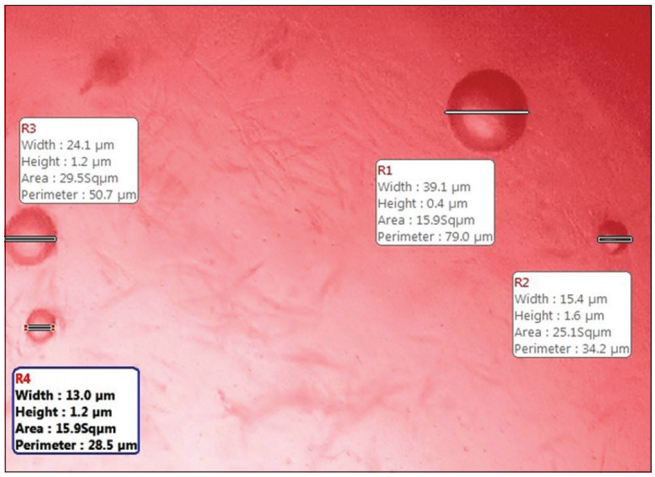

Figure 1: Digital micrograph of optimized formulation (F30)

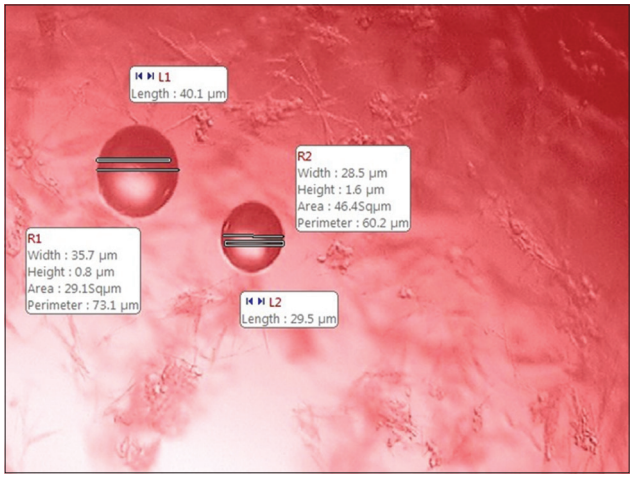

Figure 2: Digital micrograph of optimized formulation (F31)

In the present investigation, we have designed, formulated, optimized, and evaluated the successful entrapment of orlistat into liposomes with respect to its physical as well as chemical characteristics. The objective of the study has been achieved by means of maximum % drug entrapment efficiency with appropriate vesicle size and other physical and chemical stability of the liposomes as well. Physical appearance, pH, density, % drug entrapment efficiency, assay, particle size, PDI, and zeta potential of initial samples of both the optimized formulations, namely F30 and F31, were satisfactory and remained satisfactory up to 6 months at long-term storage conditions (2–8°C). Both the trials were stable up to 1 month at accelerated conditions (25°C/60% RH), but there was significant change observed after 2 and 3 months in physical as well as chemical parameters.

To protect from light and degradation, the drug was encapsulated in the form of liposomes, thereby improving the stability of the formulations. Optimization studies revealed the lipid and cholesterol concentration significantly affect the drug entrapment and size of the vesicles. There was significant change observed in some potential formula and process optimization trials during stability studies at 2–8°C up to 3 months.

From the study, it can be concluded that the ethanol injection method is suitable and acceptable process for the manufacturing of orlistat liposomes and to be stored at 2–8°C.

ACKNOWLEDGMENT

The authors would like to thank the management of AVTOS Life Sciences Pvt. Ltd., Navi Mumbai, India, for continuous support and encouragement throughout this work.

REFERENCES

1. Gutierrez de Rubatcava C, Rodriguez JL, Duro R, Alvarez-Lorenzo C, Concheiro A, Seijo B. Interaction between liposomes and hydroxypropylmethyl cellulose. Int J Pharm 2000;203:99-108.
2. Grit M, Zuidam NJ, Crommelin DJ. In Liposome Technology. Boca, Raton: CRC Press; 1993. p. 455-87.
3. Cliff RO, Ligler F, Goins B, Hoffmann PM, Spielberg H, Rudolph AS. Liposome encapsulated hemoglobin: Long-term storage stability and *in vivo* characterization. Biomater Artif Cells Immobilization Biotechnol 1992;20:619-26.
4. Grit M, Crommelin DJ. The effect of aging on the physical stability of liposome dispersions. Chem Phys Lipids 1992;62:113-22.
5. Fang JY, Lin HH, Hsu LR, Tsai YH. Characterization and stability of various liposome-encapsulated enoxacin formulations. Chem Pharm Bull 1997;45:1504-9.
6. Slabbert C, Plessis LH, Kotze AF. Evaluation of the physical properties and stability of two lipid drug delivery systems containing mefloquine. Int J Pharm 2011;409:209-15.
7. Mowri H, Nojima S, Inoue K. Effect of lipid composition of liposomes on their sensitivity to peroxidation. J Biochem 1984;95:551-8.
8. Grit M, Zuidam NJ, Underberg WJ, Crommelin DJ. Hydrolysis of partially saturated egg phosphatidylcholine in aqueous liposome dispersions and the effect of cholesterol incorporation on hydrolysis kinetics. J Pharm Pharmacol 1993;45:490-5.
9. Frokjaer S, Hjorth EL, Worts O. Stability testing of liposomes during storage. In: Liposome Technology: Preparation of Liposomes. Boca, Raton: CRC Press; 1984. p. 235-45.
10. Lichtenberg D, Barenholz Y. Liposomes: Preparation, characterization and preservation, Methods Biochem Anal 1988;33:337-462.
11. Kamel KM, Khalil IA, Rateb ME, Elgendy H, Elhawary S. Chitosan-coated cinnamon/oregano-loaded solid lipid nanoparticles to augment 5-Fluorouracil cytotoxicity for colorectal cancer: Extract standardization, nanoparticle

optimization and cytotoxicity evaluation. J Agric Food Chem 2017;65:7966-81.
12. Alexander A, Ajazuddin, Patel RJ, Saraf S, Saraf S. Recent expansion of pharmaceutical nanotechnologies and targeting strategies in the field of phytopharmaceuticals for the delivery of herbal extracts and bioactives. J Control Release 2016;241:110-24.
13. Pinilla CM, Norena CP, Brandelli A. Development and characterization of phosphatidylcholine nanovesicles, containing garlic extract, with antilisterial activity in milk. Food Chem 2017;220:470-6.
14. Lamichhane N, Udayakumar TS, D'Souza WD, Simone CB 2nd, Raghavan SR, Polf J, *et al*. Liposomes: Clinical applications and potential for image-guided drug delivery. Molecules 2018;23:1-17.
15. Vemuri S, Rhodes CT. Preparation and characterization of liposomes as therapeutic delivery systems: A review. Pharm Acta Helvetiae 1995;70:95-111.
16. Chatterjee S, Banerjee DK. Preparation, isolation, and characterization of liposomes containing natural and synthetic lipids. Methods Mol Biol 2002;199:3-16.
17. Voinea M, Simionescu M. Designing of "intelligent" liposomes for efficient delivery of drugs. J Cell Mol Med 2002;6:465-74.
18. Kirby C, Gergoriadis G. Dehydration-rehydration vesicles: A simple method for high yield drug entrapment in liposomes. Biotechnology 1984;2:979-84.
19. Kulkarni SB, Betageri GV, Singh M. Factors affecting microencapsulation of drugs in liposomes. J Microencapsul 1995;12:229-46.
20. Kannel WB, D'Agostino RB, Cobb JL. Effects of weight on cardiovascular disease. Am J Clin Nutr 1996;63:419-22.
21. Doucet E, Tremblay A. Food intake, energy balance and body weight control. Eur J Clin Nutr 1997;51:846-55.
22. Ballinger A, Peikin SR. Orlistat: Its current status as an anti-obesity drug. Eur J Pharmacol 2002;440:109-17.
23. Lucas KH, Kaplan-Machlis B. Orlistat—a novel weight loss therapy. Ann Pharmacother 2001;35:314-28.
24. Janbandhu N, Mogre R. Design, development and evaluation of orlistat liposomal drug delivery systems. Inventi Rapid NDDS 2018;2:1-9.
25. Dubey A, Prabhu P, Beladiya K, Nair N, Ghate V. Development and investigation of timolol maleate and latanoprost combination liposomes for the treatment of glaucoma. Int Res J Pharm 2015;6:256-64.
26. Elmoslemany RM, Abdallah OY, El-Khordagui LK, Khalafallah NM. Propylene glycol liposomes as a topical delivery system for miconazole nitrate: Comparison with conventional liposomes. AAPS Pharm Sci Tech 2012;13:723-1.
27. Sebaaly C, Charcosset C, Stainmesse S, Fessi H, Greige-Gerges H. Clove essential oil-in-cyclodextrin-in-liposomes in the aqueous and lyophilized states: From laboratory to large scale using a membrane contactor. Carbohydr Polym 2016;138:75-85.
28. Gharib R, Auezova L, Charcosset C, Greige-Gerges H. Drug-in-cyclodextrin-in-liposomes as a carrier system for volatile essential oil components: Application to anethole. Food Chem 2017;218:365-71.
29. Limsuwan T, Amnuaikit T. Development of ethosomes containing mycophenolic acid. Procedia Chem 2012;4:328-35.
30. Xue YY. Modern Pharmaceutics (Ping Q. N.). Beijing, China: China Med-Tech Science Beijing; 1998. p. 588-621.
31. Takeuchi H, Yamamoto H, Toyoda T. Physical stability of size controlled small unilameller liposomes coated with a modified polyvinyl alcohol. Int J Pharm 1998;164:103-11.
32. Hiemenz PC, Rajagopalan R. Principles of Colloid and Surface Chemistry. New York: Marcel Dekker; 1997. p. 120-56.
33. Kibat PG, Stricker H. Storage stability of dispersions of soyabean lecithin liposomes. Pharm Ind 1986;48:1184-9.
34. Grit M, Crommelin DJA. The effect of aging on the physical stability of liposome dispersions. Chem Phys Lipids 1992;62:113–22.

Source of Support: Nil. **Conflict of Interest:** None declared.