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Evaluation and Clinical Comparison Studies on Liposomal and Non-Liposomal Ascorbic Acid *(Vitamin C)* and their Enhanced Bioavailability

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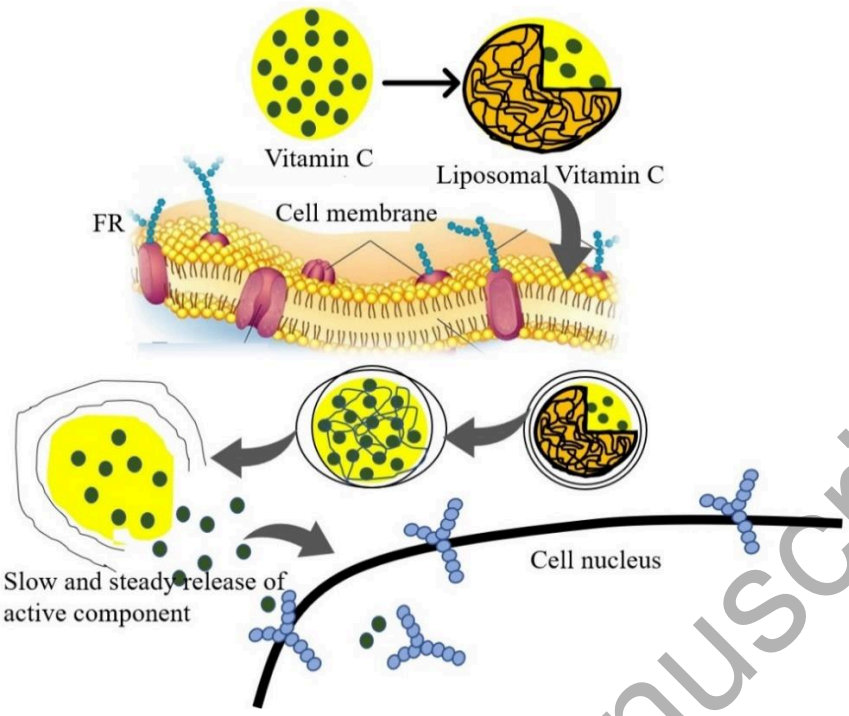
Graphical Abstract

The aim of present study was to evaluate the oral bioavailability of liposomal vitamin C and non-liposomal vitamin C in healthy, adult, human subjects under fasting conditions through an open label, randomized, single dose, two-treatment, two sequence, two-period, two way crossover, study. The vitamin C loaded liposome was well characterized using transmission electron microscopy (TEM), dynamic light scattering (DLS) and zeta potential measurements for evaluating morphology, particle size and stabilities, respectively. Microscopic image shows the core type structure which confirms the characteristic pattern of liposome. The encapsulation efficiency (EE%) and the particle size were 65.85± 1.84% and below 100 nm respectively. The results of the clinical studies of liposomal vitamin C by oral delivery to be 1.77 times more bioavailable than non-liposomal vitamin C. The Liposomal Vitamin C demonstrated higher values of C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ related to non-liposomal Vitamin C due to liposomal encapsulation. No adverse events were reported. It could be concluded that, liposomal encapsulated ascorbic acid (vitamin c) shows well organized morphological pattern, uniform particle size and highly efficient which leads to have enhanced bioavailability.



1. Introduction

The demands of various food and nutraceutical industries seek to develop products with high nutritional value by the fortification of vitamins with the aim to improve physiological function and to provide health benefits. One of the main vitamins used for this purpose is Vitamin C (Ascorbic Acid). Vitamin C is an important water-soluble vitamin widely applied in food. It is used, next to its properties as an essential vitamin, as an additive primarily due to its reducing and antioxidant properties. The use of ascorbic acid in food is related to various biological activities like protection of oxidizable compounds, retarding enzymatic browning, oxygen scavenging, inhibition of nitrosamine formation, etc. The high reactivity of vitamin C and the susceptibility to oxidation can result in fast exhaustion in various food products. The initial concentration of vitamin C sis difficult to sustain during products shelf life due to its reactive behavior. The speed of degradation of vitamin C is a complex process and depends on many variables such as relative humidity, pH, temperature etc (Assadpour & Mahdi Jafari, 2019; De Britto et al., 2016; Haham et al., 2012; Katouzian & Jafari, 2016). Nanoencapsulation technique has been widely used nowadays to enhance bioavailability and shelf life of bioactive components. Safoura et al,(Akbari-Alavijeh et al., 2020) studies the uses chitosan as a nanocarrier to encapsulates active food components. Recent years, consumers prefer to use nutraceutical supplements that can contribute to prevention/healing of various diseases and as an immunity booster. So, researchers all over the world have studied the bioavailability, efficacy and toxicity of nutraceutical supplements. Most common form of delivery is orally and it involves various key steps (1) the release of active nutraceutical ingredients in gastrointestinal fluid, their solubilization and contact with other gastrointestinal fluids (2) absorption of active nutraceutical ingredients by the epithelial

cells and their biochemical transformation. These steps are endogenous factors that are greatly influenced the bioavailability of nutraceutical products. In addition to this factor, several other factors like physicochemical properties of active components, food processing, storage etc. also matters.

Liposomes are vesicles that are simple imitated of highly complex cell membranes, comprising lipid bilayers surrounding aqueous core, which have fascinated significant attention on delivering and protecting of both hydrophilic and hydrophobic compounds like vitamins due to their unique features of biocompatibility and biodegradability. Accordingly, liposomes can promote the protection and activity of the vitamin C (Dima et al., 2020)(W. Liu et al., 2017). Liposomes comprised of biocompatible biodegradable material and it consists of aqueous volume entrapped by one or more bilayers of natural/ synthetic lipids (Sharma & Sharma, 1997; Tiwari et al., 2012). Water insoluble drugs are typically incorporated into the lipid membrane of the liposome and encapsulation is restricted by the drug-to-lipid mass ratio (Allen & Cullis, 2013; Chonn & Cullis, 1995; Nedovic et al., 2011; Wijetunge et al., 2020). Further, hydrophobic drugs can interfere with the bilayer structure of the nanocarrier, leading to liposomal instability and uncontrolled release (Akbarzadeh et al., 2013; C. Silva et al., 2012; Wu et al., 2020).

The blend of liposomal formulation of vitamin C with squeezed orange didn't change its organoleptic qualities and indicated microbiological solidness after sanitization and capacity at 4 °C for 37 days (Marsanasco et al., 2011) . A polyelectrolyte delivery system for vitamin C was accomplished by successive deposition of positive chitosan and negative sodium alginate onto the surface of anionic nanoliposomes which demonstrated that liposomes can give a potential platform for tailored design of carriers for nutrients or

preservatives to enhance both the shelf-life and safety of food matrices (W. Liu et al., 2017). Chitosan-coated nano-size liposomes were made from phosphatidylcholine (pc) and cholesterol (chol) and were promising Vitamin C carriers with a great loading efficiency and payload with 15 weeks storage over 85% Vitamin C was protected against oxidation (N. Liu & Park, 2010). Vitamin C (Ascorbic acid) is better considered as a true vitamin because in humans cannot be able to synthesis it. Vitamin C acts as a reducing agent since it exhibits a number of enzymatic/non-enzymatic effect and its ability to donate electrons. Vitamins also perform as a co-factor for a number of enzymes including collagen hydroxylation, prevents oxidative damage to DNA, intracellular proteins. In plasma it increases endothelium-dependent vasodilatation and lowers extracellular oxidants from neutrophils. Insufficiency in vitamin C results in the potentially fatal disease scurvy, low invulnerability which can be cured only by administering right dose of vitamin C (Hemilä & Chalker, 2020; Van der Velden, 2020).

Only few studies were published showing the effect of liposomal encapsulation on the bio-availability of Vitamin C. Hickey et al (2008), found in a very small study with only two persons no difference in the bioavailability of liposomal vitamin C compared to vitamin C tablets in a dosage of 5 grams. However in liposomal vitamin C consist of higher dosages such as 20 g and 36 g, plasma level of vitamin C resulted in plasma levels higher than ever shown before in literature. This result indicates that liposomes might be an excellent carrier for Vitamin C to achieve higher blood levels of Vitamin C which cannot be reached with other dosage forms. This is very important for reaching therapeutic plasma levels of Vitamin C. (Hickey et al., 2008). Davis et al. (2016) evaluated the plasma levels of oral, intravenous and oral liposomal Vitamin C. The results indicated that liposomal vitamin C have enhanced

bioavailability than non-liposomal vitamin C, while avoiding the risks associated with intravenous administration (Davis et al., 2016). However, no real bio-availability study for the absorption of liposomal vitamin C is published (Davis et al., 2016). The aim of this study is to fill this gap. An open label, randomized, single dose, two-treatment, two sequence, two-period, two way crossover, oral bioavailability study is shown for liposomal vitamin C 1000mg/5mL compared to non-liposomal vitamin C 1000mg/5mL in healthy, adult, human subjects under fasting conditions.

2. Materials and methods

2.1. Study materials

The study products non-liposomal vitamin C (NLVC) and liposomal vitamin C (LVC) were supplied by CureSupport, Deventer, The Netherlands. Liposomal vitamin C was prepared using the thin-film evaporation method. Sodium ascorbate and phospholipids were mixed in the ratio of about 2:1 (w/w) and mixed with ethanol. The suspension was evaporated by using a rotary evaporator to form a dry slurry after evaporation of the solvent (ethanol) for the phospholipids. Sodium ascorbate was dissolved as much as possible in water and stirred with the phospholipid film under high shear mixing. The concentrated suspension was homogenized under high pressure and diluted till a concentration of 1 g vitamin C per 5 mL. The material was sieved through a 0.2 µm filter. The particles lower than 200 nm were used. The end concentration was 5 g of vitamin C as ascorbic acid and sodium ascorbate per 25 mL. Non-liposomal vitamin C was prepared using the same steps as mentioned above without phospholipids. Also, this product contains 1 g vitamin C per 5 mL.

2.2. Characterisations of Liposomal Vitamin C (LVC)

2.2.1. Morphology and Particle Size Determination

Transmission electron microscopy ((TEM) JEOL-2100 model) was used to establish the morphology and dimension of LVC. Prior to analysis, dilute suspension of liposomal vitamin C pasted on a copper grid and dried in the presence of UV lamp. The analysis was carried out with accelerating voltage of 25 kV. The particle size of LVC was analysed by dynamic light scattering (DLS) analysis using a Zetasizer Nano ZS (Malvern Instruments Ltd, United Kingdom) device (S. Gopi et al., 2018). A few drops of LVC dispersion pipetted out using a micropipette, then poured into a quartz cuvette followed by dilution using Milli-Q water. The measurements were conducted at 25 °C and triplicate of each sample was measured in successive runs and in each run the sample was scanned ten times, allowing one to produce accurate size determination results (S. Gopi et al., 2018).

2.2.2. Zeta Potential Analysis

The surface charge and stability of LVC dispersions were determined by zeta potential measurements, which were conducted on the same instrument such as Zetasizer Nano ZS (Malvern Instruments Ltd, United Kingdom). The measurements were performed at 25 °C (S. Gopi et al., 2018).

2.2.3. Encapsulation Efficiency Percentage (EE%)

The supernatant obtained after centrifugation of liposomes was analysed for unentrapped 5FU using high performance liquid chromatography (HPLC, Waters, India). The experiment demonstrated out on C18 column at 30^{O}C. The EE% was calculated using equation 1(Moghimipour et al., 2018b)

$$EE\% = 100\frac{TD - FD}{TD} \qquad (1)$$

Whereas, TD is the amount of vitamin C initially added to the formulation and FD is the amount of the free vitamin C in the supernatant after centrifugation (Moghimipour et al., 2018b)

2.3. Clinical examination

2.3.1 Ethical approval

The study was commenced after a written approval obtained from the Institutional Ethics Committee. The experiments were demonstrated based on the ethical guidelines as per the biomedical research on Human Participants, ICMR (2006), ICH (Step 5) 'Guidance on Good Clinical Practice', Schedule Y (amended version, 2014) (Bhattacharya & Sur, 2007) of Central Drugs Standard Control Organization (CDSCO), 'Good Laboratory Practice', 'Good Clinical Practices for Clinical Research in India' Guidelines, Good Clinical Laboratory Practice (GCLP), Declaration of Helsinki (Fortaleza, October 2013), 21CFR part 50, 56 and 320-USFDA guidelines for industry and other regulatory requirement.

2.3.2. Study design

This study was an open label, randomized, single dose, two-treatment, two sequence, two-period, two-way crossover, oral bioavailability study in healthy, adult, human subjects under fasting conditions (Tohen et al., 2000). The flow chart of the study design was shown in Fig. 1. The aim of the analysis was to evaluate the oral bioavailability of liposomal vitamin C 1000mg/5mL (LVC), and non-liposomal vitamin C 1000mg/5mL (NLVC), in healthy, adult, human subjects under fasting conditions and to monitor the adverse events and ensure safety of the study subjects (Esfandyari et al., 2006).

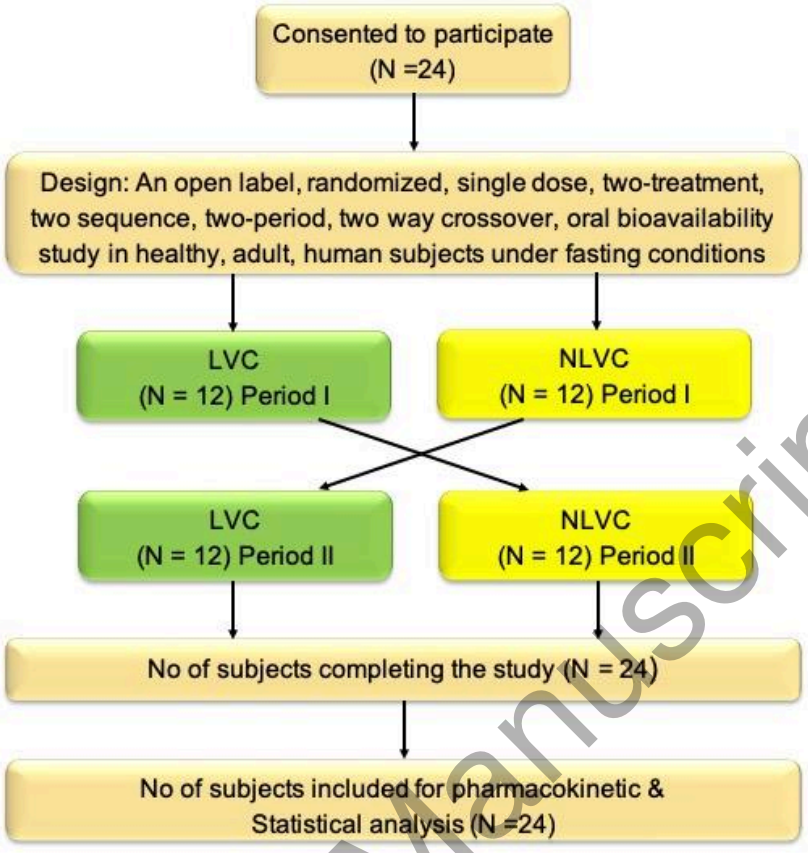


Fig. 1. Flow chart of the study design of the study

2.3.3 Inclusion and exclusion criteria

The study demonstrated with adult and healthy human beings and all registered subjects were fulfilled the experiment. The qualified persons (subjects), satisfied the inclusion/exclusion criteria for the study (Saravanan, 2014), were allocated randomly. Randomization was carried out for 24 subjects using SAS® software version 9.4. The duration of this study was 10 days (from the day of check-in of first period to the last blood sample collection of second period including a washout period of 7 days. The study was conducted at SPINOS Life Science and Research Private Limited, Coimbatore, India.

2.3.4. Dosage and style of administration

The single oral dose of the LVC or the NLVC in sitting posture with about 240 mL of water on Period I and Period II at ambient temperature (the procedure explained elsewhere (Saravanan, 2014) in a treatment time of one week were administrated to subjects under fasting conditions (Tippabhotla et al., 2013). Subsequently, the oral cavity of subjects was examined by qualified personnel. To generate randomization schedule SAS® software (version 9.4) were used after receiving the test and reference materials. The randomization was adjusted, and the code was held under controlled access (Saravanan, 2014).

2.3.5 Methodology

Totally 24 subjects were enrolled in the study. In regular intervals, subjects were housed in the clinical facility for at least 11 h pre-dose to 24 h post-dose and the washout period of at least 7 days from the successive dosing day. In every period, an overnight fasting of at least 10 h, in the beginning of day, a single oral dose, 5 mL of either LVC or NLVC was administered by using syringe, (as per randomization schedule) after administration of investigational medicinal products, syringe was cleaned with 240 mL of drinking water, at ambient temperature, to the subjects in sitting posture, under the supervision of investigator and trained study personnel (Jain, n.d.) including quality assurance auditor. Mouth check with the help of tongue depressor to assess compliance to dosing was carried out.

Totally 21 blood samples were collected including the pre-dose sampling in each period. For the pre-dose blood sample (0 h) 6 mL was collected within 60 min prior to dosing. Post dose blood samples 6 ml was collected at 0.25, 0.50, 0.75, 1.00, 1.50, 2.00, 2.50, 3.00, 4.00, 5.00, 6.00, 7.00, 8.00, 9.00, 10.00, 11.00, 12.00, 14.00, 16.00 and 24.00 hours. All samples were collected in pre-labeled K_2EDTA– vacutainers from a fore-arm vein using an indwelling cannula

as per the discretion of investigator. Heparin-lock technique was used to prevent clotting of blood in the indwelling cannula (Sreeraj Gopi et al., 2017). Before the blood sample was drawn via the indwelling cannula, 0.5 mL of blood was discarded so as to prevent the saline diluted blood and heparin (10 IU/mL) from interfering with the analysis. Cannula was removed after 24 h sample was drawn or earlier or if blocked. Vacutainers were positioned vertically in a stack kept in ice bath while centrifugation and during separation. After assortment of blood tests from all the subjects at each time point, the gathered examples were put in a thermo-protected box containing wet ice and it was moved to the sample preparing room where the blood tests was centrifuged at 4000 ± 50 rpm for 10 minutes at 2 °C to 8 °C to isolate the plasma. Centrifugation was carried out within 30 min of the collection of samples, at each time-point. The separated plasma was equally transferred to polypropylene tube (previously labeled with study code and sample code) in two aliquots. These aliquots were stored upright at a temperature of -30 ± 10 °C and -70 ± 15 °C, till the end of the study. At the end of the study, the samples were transferred to the bioanalytical facility in a thermo-insulated boxes containing sufficient dry ice. Plasma vitamin was observed by ultra-performance liquid chromatography (UPLC). Subjects were supplied with standard diet and continuously examined for well-being and safety throughout the study. Quality assurance audits were conducted at different phases of study to ensure that the study was performed in accordance with good clinical and laboratory practices and applicable regulatory norms.

2.3.6 Diagnosis and main criteria for inclusion and exclusion

Subjects who satisfy all of the following conditions were considered for study enrollment, which are included normal, healthy, adult, male and female subjects of age between year 21-65 with a body mass index (BMI) in between 18.50 kg/m^2 to 24.99 kg/m^2 (Jude et al., 2018; Lopez-

Toledano et al., 2017; Maki et al., 2018; Saravanan, 2014). Subject agreed avoid vitamin C containing medicines and dietary supplements from screening until last visit and high activity physical exercise 72 h prior to last visit. Subjects having no proof on fundamental sickness at the time of screening followed by registration and their screening is conducted inside 29 days of check in. The values from the screening lab are inside ordinary cutoff points or considered by the doctor or head/clinical examiner to be of no clinical noteworthiness. Sound as reported by the clinical history, physical assessment (including but not limited to assessment of the gastrointestinal, respiratory, cardiovascular, musculoskeletal and focal sensory systems) and imperative sign appraisals. Typically, healthy as documented by 12-lead electrocardiogram (ECG), X-Ray and clinical research center appraisals. Non-smokers or ex-smokers, the ex-smokers are characterized as somebody who has totally quit smoking for at any rate the previous 3 months. Subjects were ready to consume ova-lacto vegetarian diet and to comply with all requirements of this study protocol as well as instructed by the study personnel.

The subjects with history or significant presence of the following were excluded from participation/enrollment in the study, which are the indication of allergic reaction or identified hypersensitivity to vitamin C or other drugs. Subjects with cholestasis, hepatic encephalopathy, history of liver disease, myasthenia, alcohol abuse, existing tinnitus, renal or liver impairment and pre-existing gallbladder disease. Any general disease during the previous 3 months or several current lasting medical diseases. Any disease or condition which might compromise the haemopoietic, gastrointestinal, renal, hepatic, cardiovascular, musculoskeletal, respiratory, central nervous system, diabetes, psychosis or any other body system. History of liquor addiction or abuse and malabsorption syndrome that influences vitamin c metabolism. Cardiovascular failure, angina pectoris, ventricular arrhythmias or atrial fibrillation with >100/min ventricular

rate. Gastrointestinal bleeding in last three months, uncontrolled diabetes mellitus, active psychiatric disorder, intention for suicidal, disorders with unconsciousness, psychopathic disorder, lack of cooperation, chronic obstructive lung disease or active smoking (more than 2 cigarettes in the past 6 months), taking more than 100 mg vitamin C daily within 14 days to screening (Moghimipour et al., 2018b) as per ClinicalTrial.gov (NCT 0012 2184). Consumption of caffeine and /or xanthine containing products (i.e. coffee, tea, chocolate, and caffeine-containing sodas, colas, etc.), tobacco containing products for at least 24 h prior to check-in and throughout the entire study.

Consumption of grapefruit and its juice and poppy containing foods (Fitzsimmons et al., 2018) for at least 72 h prior to check-in and throughout the study. Subjects who taken any prescription medications, over the counter medical products, herbal medications within 14 days prior to study in and throughout the study. History of dehydration from diarrhea, vomiting or any other reason within a period of 24 h prior to study check-in of each period, an unusual or abnormal diet within 48 h prior to study check-in of each period, for whatever reason because of fasting due to religious reasons. Positive results for drugs of abuse (Marijuana-THC, amphetamine-AMP, barbiturates-BAR, cocaine-COC, benzodiazepines-BZD and morphine-MOR) in urine and positive results for alcohol breath test prior to check-in of this study period. Any blood donation or excess blood loss within 90 days of check in, systolic blood pressure less than 90 mmHg or more than 140 mmHg and diastolic blood pressure less than 60 mmHg or more than 90 mmHg. Intake of any kind of hormonal agent at any time within 2 weeks prior to start of study check in (Saravanan, 2014).

2.3.7 Safety evaluation

Safety evaluations were done based on clinical perceptions, laboratory data at the start and at the end of the investigation and assessment of the adverse events observed during the course of the study (S. L. Rogers et al., 2000; Saravanan, 2014). All study participants were continuously monitored by medical personnel during the housing periods. A physician was available during the study periods. Clinical examinations and vital signs measurements were done before check-in, before check-out (24.00 h), at pre-dose (0 h), at 01.00, 03.00, 06.00 and 12.00 hours after dosing were documented in the case report forms.

2.3.8 Pharmacokinetic evaluation

The study was considered to evaluate comparative bioavailability of LVC and NLVC, consequently pharmacokinetic profile (rate and extent of drug) of both LVC and NLVC products were assessed based on measured concentration of drug in human plasma collected in clinical phase (Saravanan, 2014). The extent of relative oral bioavailability value for AUC_{0-t} was found using the formula of AUC_{0-t} of LVC / AUC_{0-t} of NLVC. The pharmacokinetic analysis was performed employing the estimated concentration vs. time profiles of Vitamin C using WinNonlin® version 7.0 (Sreeraj Gopi et al., 2017). The parameters, AUC_{0-t}, C_{max} and $AUC_{0-\infty}$ were considered as primary pharmacokinetic parameters and t_{max}, $t_{½}$, and K_{el} were considered as secondary pharmacokinetic parameters.

2.3.9 Statistical analysis

Analysis of variance (ANOVA) consistent with two one-sided test for bioequivalence, ratio analysis and 90% confidence intervals for ratio of least square mean of Ln-transformed data of C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ for vitamin C were calculated by using SAS® software version 9.4 of SAS Institute Inc, USA.

3. Results and discussion

3.1. Characterisations of Liposomal Vitamin C (LVC)

3.1.1 Transmission Electron Microscopic analysis

The use of liposome as a nanodrug delivery carrier should satisfy following criteria (1) the amount of drug encapsulated in liposomes, (2) particle size and (3) stability of liposome (Moghimipour et al., 2018b; Pereira et al., 2016). To better understand the morphology of drug encapsulated liposome, TEM studies were conducted (Fig. 2).

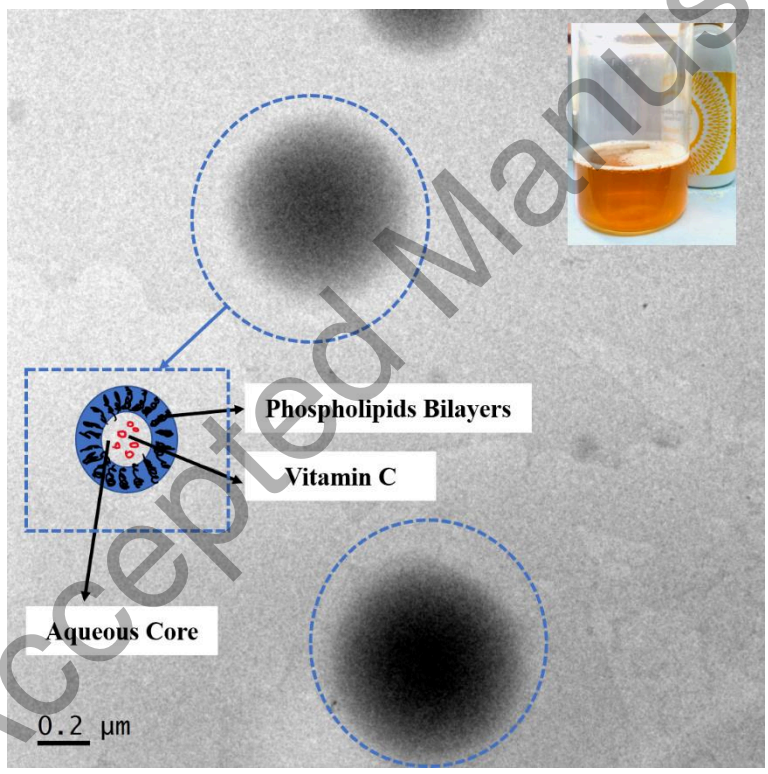


Fig. 2. TEM image of Vitamin C loaded liposomal product

By closely look into the TEM images, we can see that the liposomes are spherical in size and shape and there was minimal liposomal debris which might be indicative of vesicle rupture (Cipolla et al., 2014). The encapsulation efficiency of Vitamin C in liposomes are found to be

65.85± 1.84% which was calculated by chromatographic technique. This encapsulation efficiency indicates that liposome are suitable drug carriers. Liposomes were spherical in shape (Bochot & Fattal, 2012; Moghimipour et al., 2018a) and are well dispersed as indicated by the TEM image (Fig. 2). The mean particle size of liposomes was calculated by *image J* software and found to be 245.00± 4.58 nm. After conjugation with Vitamin C, the spherical morphology of liposomes, remain unaltered. It is well known that the size of liposomes is one of main influence for the tissue targeting. Larger liposomes are often taken up by phagocytes; while, small liposomes (100 to 200 nm) can penetrate easily in tumor tissue due to higher permeability and retention (EPR) effects (Maione-Silva et al., 2019; Moghimipour et al., 2018a; Nogueira et al., 2015).

3.1.2 DLS and zeta potential analysis

Even morphology of liposomes was confirmed by TEM images, the size distribution and geometry of nanoliposomes cannot be fully understood as very small fraction of sample is being analysed. The principle of DLS is based on scattered light intensity caused by the Brownian movement of particles and it calculated the hydrodynamic volume of suspension (Balakrishnan et al., 2018). Fig. 3a shows intensity distribution data of liposomal vitamin C. The results suggest that maximum intensity of nanoliposomes lies below 100 nm. Usually liposomes have higher polydispersity index (PDI) which suggest a heterogenous vesicle population. The results of particle size measurements by DLS method indicated that liposomes have a homogenous size distribution (Karn et al., 2013).

Stability is one of the critical factors to be considered during the formulation and design of nutraceutical formulation. Physical instability of liposome formulations is mainly due to the

increase in particle size due to self-aggregation of liposome caused by the processing methods and/or long term storage. Gradually this instability results in rapid uptake of drugs by the epithelial system with subsequent rapid clearance and shorter lifetime. Hence preparing liposomal nanostructures with uniform size and stable lifetime is one of the important facet of developing nutraceutical formulations/products (Karn et al., 2013).

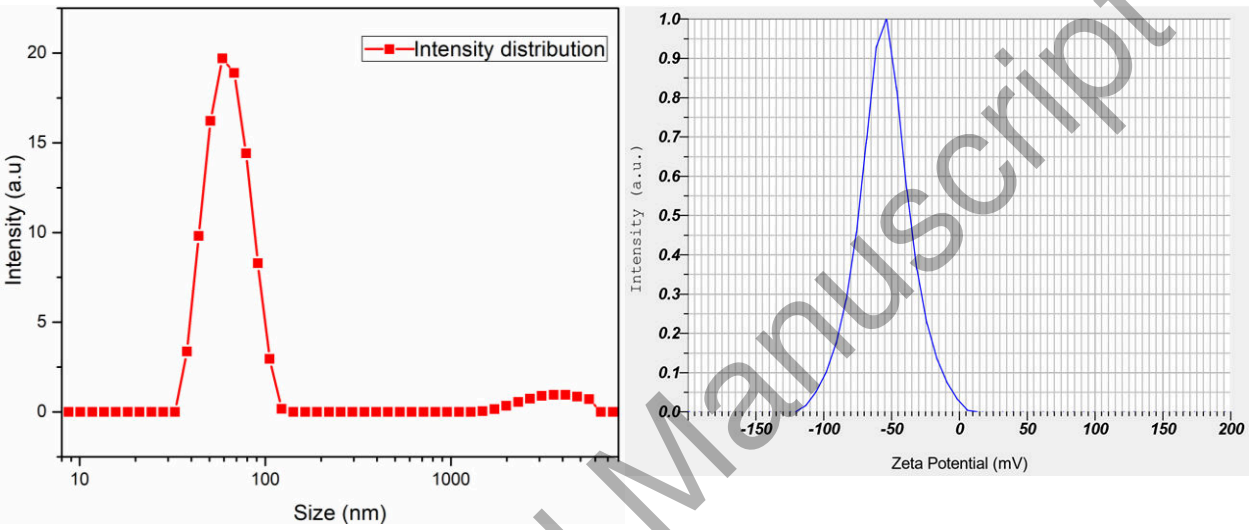


Fig. 3. (a) DLS (Intensity distribution data of LVC) (b) Zeta potential of LVC.

Fig.3b shows zeta potential value of LVC. The zeta potential is calculated based on velocity and direction of particles under the influence of a known electric field (Pignatello et al., 2006). It illustrates the charge of the particles (surface), and used to measure the possible extent of flocculation and de-flocculation and colloidal stability of nanosuspension (IM & 2003, n.d.). Generally, a large positive or negative value of zeta potential indicates better physical stability of colloidal suspension due to electrostatic repulsion between individual particles(Joseph & Singhvi, 2019). Small zeta potential value results in particle aggregation and flocculation due to van der waals force of attraction exists between them which results in

physical instability of colloidal suspension. In the present study, zeta potential have greater value (-40 mV) (Hakkimane et al., 2018). This confirms that our formulation on LVC is highly stable and contains non-agglomerated LVC.

Polymeric encapsulation (liposomal) provides protection to active nutraceutical ingredients from surrounding conditions due to the possibility of more drug reaching the targeted site. Since LVC are taken up by the cells by a different mechanism called endocytosis and able to release active ingredient ie, vitamin C in a slow, sustained and steady manner inside the cellular membrane and they can deliver the therapeutic amount of drug to kill the intracellular bacilli (Hakkimane et al., 2018)(J. A. Rogers & Anderson, 1998).

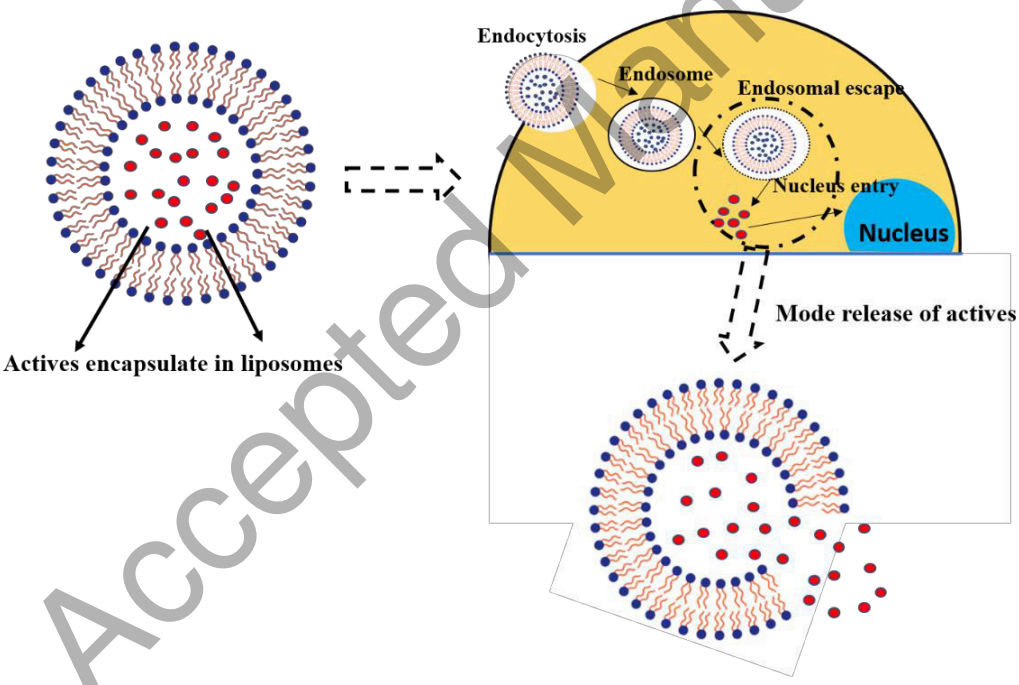


Fig. 4. Mode of delivery of active ingredients

To deliver active ingredients the liposome must be circulated inside the body for a considerable amount of time (Roy et al., 2015). Main mechanism behind the interaction of

active ingredient loaded liposomal structure is Endocytosis. The endosomal lipidic layer components destabilizes after the entrance of LVC complex into the cells (Fig. 4). Endosomal liposomal structure gradually diffuses into liposomal complex and form neutral ion pairs. This eventually release displacement of actives from liposomal structure and release into cytosol (Mazidi et al., 2016).

3.2 Clinical experiments

All 24 subjects finished both the periods of the study. Their demographic details are given in Table 1. Their average age, height, body weight and BMI were 33.54 years, 170.25 cm, 75.08 kg and 25.86 kg/m^2 respectively. Here, one thing to be noted that, all subjects in the study were Asian. The vital signs of the subjects were comparable throughout the study. There is no oppositional experience reported throughout the course of the analysis. Therefore, it can be assumed that both the LVC and NLVC samples were nontoxic and well accepted at the selected dose level in the human subjects. The LVC and NLVC products were administrated in a randomized schedule. The main objective of this study was to evaluate the oral bioavailability of LVC compared to NLVC.

Parameter	Mean	SD	Min	Max	CV%
Age (years)	33.54	6.53	23.00	43.00	19.48
Height (cm)	170.25	5.79	157.00	178.00	3.40
Weight (Kg)	75.08	9.64	61.00	95.00	12.83
BMI (Kg/m^2)	25.86	2.62	21.30	29.98	10.14

Table 1. Demographic characteristics of study subjects

A substantial difference in the absorption of vitamin C in LVC was observed as compared to NLVC (Fig. 5). The parameters of individual pharmacokinetic for each subjects based on the treatment for vitamin C, analysis of variance was completed on the Ln-transformed data of C_{max}, AUC_{0-t}, $AUC_{0-\infty}$, t_{max}, $t_{½}$, and K_{el} . The results are given Table 2. In addition to this, the 90% confidence intervals and intra-subject CV% was determined for these parameter (Table 3).

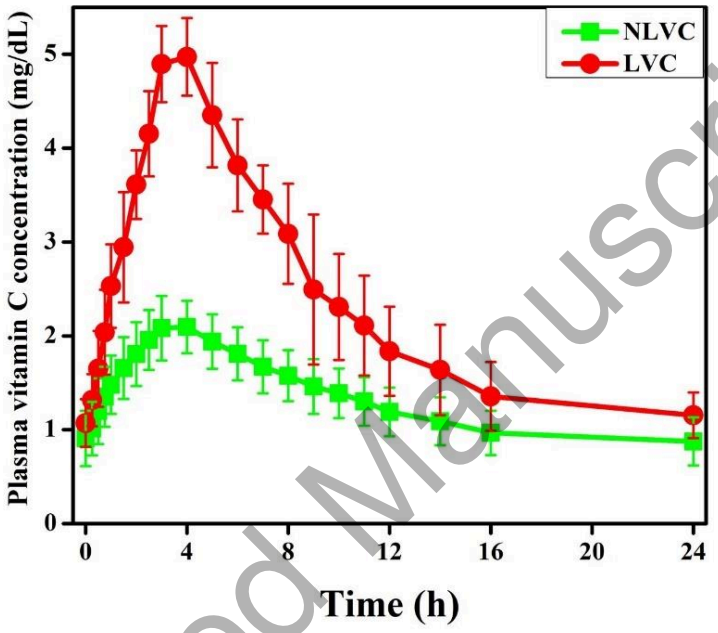


Fig. 5. Mean plasma concentration (mg/dL) of vitamin C form LVC compared with NLVC. All the values are reported in mean ± SD

Pharmacokinetic Parameter	LVC	NLVC
C_{max} (mg/dL)	5,23	2.17
AUC_{0-t} (mg.h/dL)	55.86	31.53
$AUC_{0-\infty}$ (mg.h/dL)	78.90	57.12
t_{max} (h)	3.51	3.42
K_{el} (1/h)	0,056	0.037
$t_{½}$ (h)	12.39	18.99

Table 2. Geometric mean of LVC and NLVC for ascorbic acid (vitamin C) (N=24)

The AUC is one of the consistent measures of the bioavailability which specifies more accurate value due to the measurement of entire response over time period [comparative paper]. The maximum plasma vitamin C (C_{max}) and AUC_{0-t} for the LVC were 5,2 mg/dL and 55,9 mg/dL respectively, whereas for the NLVC were 2,2 mg/dL and 31,5 mg/dL. The extent of the relative oral bioavailability of vitamin C was found higher for LVC than NLVC, which is indicated that the LVC was shown 1.77 times more bioavailable than the NLVC and 2.41 times better considering of the rate of absorption.

A significant difference ($P < 0.0001$) was observed between LVC and NLVC products due to the treatment effect for the pharmacokinetic primary parameters C_{max}, AUC_{0-t}, $AUC_{0-\infty}$ (Nazarudheen et al., 2013), but there was no significant difference between LVC and NLVC products in the sequence and period effect (Table 3).

Pharmacokinetic Parameter	Geometric Least Square Mean		P value			Intra Subject CV (%)	Ratio (%)	90% Confidence Interval	Power (%)
	LVCC	NLVCC	Treatment	Sequence	Period				
C_{max} (mg/dL)	5.2386	2.1695	< 0.0001	0.4986	0.2686	8.19	241.47	231.88 - 251.46	100.0
AUC_{0-t} (mg.h/dL)	55.8627	31.5286	< 0.0001	0.0045	0.0228	13.32	177.18	166.14 - 188.95	99.99
$AUC_{0-\infty}$ (mg.h/dL)	78.9010	57.1165	< 0.0001	0.1080	0.1430	23.66	138.14	123.05 - 155.08	93.75

Table 3. Statistical results of LVCC versus NLVCC for vitamin C as ascorbic acid

The first bioavailability study of vitamin C was reported by Hickey et al. in 2008. Hickey et al. proved that there is no difference in absorption between 5 g liposomal vitamin

C as compared to 5 g of non-liposomal vitamin C in commercial tablets. It needs to be remarked that Hickey used tablets with Bioflavonoids which might influence absorption of Vitamin C. Hickey also showed that the consumption of 20 and 30 g of liposomal vitamin C registered remarkable increase in plasma levels of vitamin C which were not shown in literature before, (Hickey et al., 2008). From this study it can be concluded that there is an advantage in using the more expensive liposomal vitamin C compared to tablets only in the high dosages (> 5 grs). Juan Manuel Serrano Nunez (2014) showed a significant bioavailability of vitamin C in rats for liposomal sodium ascorbate as compared with non-liposomal sodium ascorbate (Sánchez-Lara et al., 2014). Davis et al. (2016) demonstrated more bioavailability of vitamin C by oral consumption of equivalent to 4 g of vitamin C, when delivered through liposomes compared with un-encapsulated vitamin C. In spite of these results Mikirova et al., found no effect of encapsulation on bio-availability of Vitamin C and concluded that liposomal encapsulation of ascorbic acid demonstrated no more effect than normal oral intake *(Liposomal Vitamin C obtained from LivonLabs; personal communication)* (Mikirova et al., 2019) (Davis et al., 2016).

It is hard to make pharmacokinetic comparisons between vitamin C formulations that contain different amounts of vitamin C (Sreeraj Gopi et al., 2017), having different formulations and are analyzed in different analytical procedures. However, it is remarkable to see that the data found in literature differ so widely. These differences are illustrated in Table 4 which summarizes published pharmacokinetic data from studies with vitamin C (Davis et al., 2016; Sreeraj Gopi et al., 2017; Hickey et al., 2008) and different liposomal vitamin C formulations used by Hickey et al (Hickey et al., 2008) Davis et al (Davis et al., 2016) and Mikirova (Davis et al., 2016).

Vitamin C dose (mg)	C_{max} (mg/dL)	C_{max} per mg Vitamin C	Reference
1000	5.24 (258 mM/L)	52.4	Current study
1000	1.35 (80 mM/L)	13.5	Levine et al. 1996 (Levine et al., 1996)
5000	4.4 (250 mM/L)	8.8	Hickey et al. 2008 (Hickey et al., 2008)
5000	4.3 (245 mM/L)	8.6	Hickey et al. 2008 (Hickey et al., 2008)
4000	3.2 (228 mM/L)	8.0	Davis et al. 2016 (Davis et al., 2016)

Table 4. Pharmacokinetic properties of various vitamin C combinations expressed per mg

The above data were compared based on C_{max} per mg vitamin C administrated. The average C_{max} per mg vitamin of the LVC product as compared with the averages of unformulated vitamin C reported by Levine et al. (Gill et al., 1995) and Hickey et al. (Hickey et al., 2008), the values of LVC were about 4 and 6 fold greater respectively. When one makes this comparison between the LVC with the value found by Hickey, the C_{max} per mg vitamin C for the LVC study product is approximately 6-fold greater. A similar comparison with the data found by Davis et al (Davis et al., 2016) indicates that the C_{max} per mg vitamin C for the LVC product was approximately 6.6 fold greater which is shown in table 4. In this study, even though the subjects were consumed 1 g equivalent vitamin C the bioavailable difference were greater, remarkable and more striking. At this point it is not possible explain these differences. It might be the differences in the pharmacokinetics of the volunteers in the different studies, the dissimilarities in the analytical procedures followed to measure plasma levels of Vitamin C or the differences in the liposomal formulations used to encapsulate

Vitamin C. The data in this study show that it is possible to increase the bio-availability of ascorbic acid by encapsulation in liposomes substantially. This means that liposomal encapsulation is absolute a promising route for the increase in the clinical effectiveness of oral ascorbic acid. However, more study is required to obtain reliable formulations and dosage regimes to obtain the clinical goals.

4. Conclusions

In summary, this study was an open label, randomized, single dose, two-treatment, two sequence, two-period, two way crossover, oral bioavailability study of Vitamin C encapsulated in liposomes in healthy, adult, human subjects under fasting conditions. Microscopic image shows the core type structure which confirms the characteristic pattern of liposome. After conjugation with Vitamin C, the spherical morphology of liposomes, remain unaltered. The encapsulation efficiency (EE%) and the particle size were 65.85± 1.84% and below 100 nm respectively. The results of the clinical study indicate that the oral delivery of vitamin C encapsulated in liposomes registered 1.77 times more bioavailable than the non-liposomal vitamin C. The liposomal vitamin C demonstrated bioavailability by improving responses of C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ related to non-liposomal vitamin C. As an illustration, normalization of the data on the basis of C_{max} per mg administrated vitamin C indicates that the LVC exhibits greater bioavailability as compared to several other studies.

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