RESEARCH PAPER

Formulation and Evaluation of Enteric-Coated Chitosan–PEG Dual Coated Liposomal Vitamin B12 for Intrinsic Factor Independent Oral Absorption

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ABSTRACT

Vitamin B12 deficiency is a considerable public health problem, especially in elderly people, vegetarians, patients with gastrointestinal disorders and those with decreased secretion of intrinsic factors. The absorption of conventional oral supplementation is poor and variable, it depends on the intrinsic factor mediated transport pathway; parenteral therapy has the drawbacks of pain, inconvenience and poor patient compliance. The purpose of the present study was to formulate and characterize a novel multi-layered oral delivery system which shows increased oral bioavailability and intrinsic factor independent absorption due to vitamin B12 loaded liposomes coated with chitosan and polyethylene glycol (PEG 4000) and enteric coated with sodium alginate. Vitamin B12 loaded liposomes were synthesized and then modified with chitosan to give them mucoadhesive and permeation enhancing properties. To impart good colloidal stability and mucus penetration, PEG 4000 was used and sodium alginate was used as an outer enteric coating for protection against gastric degradation and subsequent intestinal release. The particle size, zeta potential, encapsulation efficiency, morphology, in vitro release profile and stability of the formulations were assessed. The developed ML liposomal system exhibited desirable physicochemical characteristics with an improved in vivo stability in the gastrointestinal tract and controlled release of vitamin B12 in the intestinal tract. The mucoadhesion and paracellular transport were improved by chitosan coating and the penetration of the mucus was enhanced by PEG 4000. The coating of sodium alginate was effective in protecting the gastric wall and responsive to changes in pH. These components also worked synergistically to aid in intestinal delivery and to potentially increase B12 bioavailability. The multifunctional nanocarrier system provides a gastrointestinal degradation protection, a prolonged intestinal residence, an enhanced epithelial permeation, and intrinsic factor-independent absorption, which are potential alternatives to conventional oral and injectable vitamin B12 therapy.

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INTRODUCTION

Vitamin B12 (cobalamin) is a water-soluble vitamin essential for DNA biosynthesis, erythropoiesis, neurologic and cellular metabolism [1]. It functions as a cofactor of enzymes that can't function without it, such as methionine synthase and methylmalonyl-CoA mutase, which are required for methylation, myelin synthesis, and fatty acid metabolism. Vitamin B12 deficiency can result in megaloblastic anemia, peripheral neuropathy, cognitive and brain dysfunction and psychiatric manifestations and cardiovascular complications [2]. The incidence of vitamin B12 deficiency is especially high in older adults, in strict vegetarians, in patients with gastrointestinal diseases and in patients on long-term proton pump inhibitor or metformin therapy [3]. Oral delivery is the most desirable way to deliver drugs because it is convenient and patients accept it. However, oral delivery of vitamin B12 is difficult due to its large molecular weight, hydrophilic properties, degradability and requirement for the intrinsic factor receptor-mediated absorption pathway. Vitamin B12 absorption is a complicated process that is interrupted by a number of pathophysiological states [4]. Traditional oral products have therefore poor and variable bioavailability and injected products have to be administered repeatedly and often fail to achieve good patient compliance. These limitations can be addressed with nanotechnology-based

delivery systems [5]. Of these, the liposomes are especially promising due to their biocompatibility, their ability to encapsulate hydrophilic molecules, the protection of the encapsulated therapeutic substance from degradation and the potential to increase the absorption of drugs in the intestine. However, the conventional liposomes are unstable in the gastric fluid, are disrupted in the presence of bile salts and have poor interaction with the mucosa [6]. In the present study a multifunctional liposomal delivery system containing chitosan, polyethylene glycol (PEG 4000) and sodium alginate, was proposed as a tool for overcoming these challenges. Chitosan displays mucoadhesive properties and short term opening of epithelia tight junctions, which promotes the permeation in the intestine [7]. PEG 4000 stabilises the colloids, prevents liposome aggregation and aids penetration of the mucus. The sodium alginate is a pH responsive enteric coating which protects the formulation in the acidic environment of the stomach and allows it to be released in the intestine [8]. A system containing these components as a single multilayered nanocarrier is supposed to facilitate the intrinsic factor independent uptake of vitamin B12 and enhance the therapeutic effect [9].

MATERIAL & METHODS

Methylcobalamin (Vitamin B12, IP) was purchased from Mahima Life Sciences Pvt. Ltd., Mumbai. The lipid bilayer forming agents used were soya lecithin and cholesterol (Research Lab Fine Chemical Industries Pvt. Ltd., Mumbai). The coating polymers were chitosan (75% deacetylation) and PEG 4000 and sodium alginate. HPLC or AR grade solvents and reagents were used. In all cases, double-distilled water was used.

Organoleptic Evaluation

Organoleptic properties are primary properties which indicate purity & identity of the drug. The desired amount of Methylcobalamin IP was deposited on a clean glass watch glass and examined visually for physical condition, colour and crystallinity under fluorescent light. The odor was tested by olfaction. The observations recorded and compared with the official compendial criteria given in Indian Pharmacopoeia (IP) [10].

Determination of Melting Point

The melting point is an important one that can be used to verify the purity of a crystalline substance. In short, one end of a glass capillary tube was closed by a flame of Bunsen burner. The Methylcobalamin IP powder was injected into the capillary in a very small amount and was gently tapped to pack the powder tightly into the bottom of the capillary that was sealed. The range of temperature which was observed compared to the theoretical range of temperature (133-155°C) [11].

UV-Visible Spectrophotometric Analysis

The correct amount of Methylcobalamin IP (10 mg) was dissolved in methanol and then diluted to a stock concentration of 100 µg/mL. A suitable dilution was scanned in the UV-visible region (200–600 nm) against a methanol blank to determine the wavelength of maximum absorbance (λmax), which was found to be 356 nm. Aliquots of the stock solution were then diluted with methanol to give standard concentrations to be used for calibration and absorbance was measured at 356 nm. A calibration curve was plotted with absorbance vs. their concentrations and linearity was evaluated by the correlation coefficient (R^2) based on the Beer–Lambert's law [12].

Drug-Excipient Compatibility Studies (FTIR)

FTIR spectroscopy was used to assess the compatibility of Methylcobalamin with formulation excipients. The KBr pellet method was used to analyze pure Methylcobalamin, Methylcobalamin–Chitosan, Methylcobalamin–PEG 4000 and Methylcobalamin–Sodium Alginate physical mixtures. Samples (2-5 mg) were blended with IR-grade KBr and pressed into pellets which were then scanned in the range 4000-500 cm^{-1}. To evaluate the compatibility between the drug and the excipients, spectra were evaluated for peak retention, peak shift and peak broadening [13-14].

Differential Scanning Calorimetry (DSC)

Differential Scanning Calorimetry was used for analyzing the thermal behavior, crystalline state and purity of Methylcobalamin IP. An accurate weight of the drug sample (about 6.7 mg) was placed directly into a standard aluminum DSC pan and was hermetically sealed with an aluminum lid. An empty sealed aluminum pan was used as a reference. Thermal analysis was performed under dynamic nitrogen atmosphere at the heating rate of 10°C/min in order to avoid the oxidative degradation in the range of temperature 0°C to 340°C [15].

Formulation Development of Liposomes

Preparation of Uncoated Vitamin B12 Liposomes

Eighth uncoated liposomal formulations (F1–F8) were prepared by ethanol injection method. The aqueous phase consisted of a 100 mL of PBS (pH 7.4) containing methylcobalamin (5 mg). The lipid phase was prepared by dissolving soya lecithin (100-200 mg) and cholesterol (20-40 mg) in 10 mL ethanol. The injection of the lipid solution was done into the aqueous phase under rapid magnetic stirring (600 or 900 rpm) which led to spontaneous formation of liposomes. Then the dispersion was sonicated for 10 min to decrease the size of the vesicles to achieve uniform liposomes. The prepared formulations were kept in amber colored glass vials at 4°C for further evaluation [16].

Preparation of Multi-Layered Coated Liposomes

The prepared optimized liposomal formulation (F3) containing Methylcobalamin (5mg), Soya Lecithin

(100mg) and Cholesterol (20mg) was selected for coating with the superior physicochemical characteristic and then coated by the layer by layer (LbL) technique. Chitosan (0.1% w/v), PEG 4000 (0.1% w/v) and Sodium Alginate (0.1% w/v) solutions were prepared in advance separately. Liposomal dispersion was initially coated with chitosan by dropwise addition under continuous stirring for 1 h to form mucoadhesive layer by electrostatic adsorption. Then dropwise addition of PEG 4000 solution was made and stirred for steric stabilization and improve mucus penetration. Finally, the dispersion was dropwise added to the sodium alginate solution and the mixture was stirred for further 1 h, before uniformly coated multilayers liposomes were obtained due to the electrostatic interaction between the chitosan layer and sodium alginate solution [17-18].

Characterization of Liposomal Formulations

Particle Size & Polydispersity Index (PDI)

The sizes of untreated (F1-F8) and coated liposomes were measured by Dynamic Light Scattering (DLS) on Malvern Zetasizer Nano ZS, which provides the hydrodynamic diameter (Z-average) and size distribution (Polydispersity Index, PDI). DLS or photon correlation spectroscopy measures the fluctuations in intensity of scattering when the Brownian motion of the particles in the suspension causes the intensity. Liposomal dispersions were appropriately diluted (usually 10- to 100-fold) in ultra-purified water to avoid any multiple scattering phenomena prior to analysis. The measurements were taken at a scattering angle of 90° at a constant temperature of 25℃. The higher the PDI, the more polydisperse and wider the range of particle sizes of the system [19].

Zeta Potential Determination

To predict the electrokinetic stability of liposomes in the long-term against aggregation, the electrokinetic potential at the slipping plane of the colloidal particles was measured as zeta potential. Electrophoretic Light Scattering (ELS) was used for measurements (Malvern Zetasizer Nano ZS). All uncoated batches were measured to achieve negative baseline stability and after multi-layer coating process to ascertain successive deposition of polymers [20].

Determination of Entrapment Efficiency (EE%)

A certain amount of liposomal dispersion was put in the centrifuge tubes and ultracentrifuged for 30 min at 4°C with high-speed cooling ultracentrifuge (15000 rpm). In such a case the intact liposomes containing the drug pellet in the bottom of the tube and the unencapsulated (free) drug will be in the supernatant. The clear supernatant was carefully decanted and suitably diluted in methanol and the concentration of free Methylcobalamin was determined by spectrophotometric measurement at 356 nm according to the calibration curve [21].

Scanning Electron Microscopy (SEM)

Both optimized uncoated (F3) and final coated liposomes were characterized by SEM which provided information on their morphological parameters, surface topography and structural integrity. A small amount of the liposomal dispersion was carefully applied to a double-sided carbon tape attached to an aluminium SEM stub. The sample was dried at room temperature until it was completely dry. In order to make the sample electrically conductive and to avoid charging artifacts of the electrons when imaging, the dried sample was coated with a thin layer of gold-palladium in a high vacuum. The coated samples were then inserted into the SEM chamber & imaged at an accelerating voltage of 20.0 kV with a working distance (WD) of 11.6 mm. The micrographs obtained were examined to ensure that the vesicles would be spherical, smooth, and would not form large aggregates [22].

***In-Vitro* Drug Release Studies**

The permeability of the sodium alginate enteric coating and the controlled release of Methylcobalamin from it were studied by using the dialysis membrane method with USP Dissolution Apparatus II, in vitro. The optimized multilayer-coated liposomal formulation was individually tested in Simulated Gastric Fluid (SGF, pH 1.2), Simulated Intestinal Fluid (SIF, pH 6.8) and Phosphate Buffer (PB, pH 7.4) for 12 h. The formulation was packed into a pre-hydrated cellulose dialysis membrane (MWCO 12,000–14,000 Da) and placed into 900 mL dissolution medium at 37 ± 0.5°C with stirring at 50 rpm. Samples of 5mL were removed from the dissolution medium at pre-determined time points and replaced with an equal volume of pre-warmed dissolution media, thus ensuring sink conditions. Drug release was spectrophotometric at 356nm and cumulative percentage drug release was determined and plotted against time [23].

Comparative *In-Vitro* Release Study of Marketed Formulation

A comparative in vitro dissolution study was conducted with a marketed Methylcobalamin tablet for comparative measurement of the release characteristics of the developed enteric-coated liposomal formulation. The same USP Dissolution Apparatus II, fitted with 900 mL of Simulated Gastric Fluid (pH 1.2), Simulated Intestinal Fluid (pH 6.8) and Phosphate Buffer (pH 7.4) operating at 37 ± 0.5°C and a paddle speed of 50 rpm were used for the marketed formulation. Samples were removed every 2 h for 12 h, substituting with fresh medium to ensure that the medium was not at the sink, and spectrophotometrically measured at 356 nm. The drug release profiles of the developed multilayer-coated liposomes were compared with the cumulative percentage drug release in the three different dissolution media to evaluate the efficacy of the enteric coating to

reduce the release of the drug in the stomach and enhance the controlled release of the drug in the intestine [24].

Stability Study of Coated Liposomes

Coated liposomal preparation stability study was performed stored at temperature of 4°C. The sample was examined for a period of more than three months for changes. The study was conducted to establish the short-term efficiency & safety with a view of getting a compact stability profile by keeping the monitoring of the changes during the three-month period [25-26].

RESULTS & DISCUSSION

Organoleptic properties

The organoleptic properties (appearance, colour & odour) of the drug sample Methylcobalamin IP were examined and the findings are reported in table 1 below.

Table 1. Organoleptic properties of Methylcobalamin IP

Test	Observation	Inference
Appearance	Dark red crystalline	Complies with IP
Colour	Dark red	
Odour	Odourless	

From results obtained in the above test, the sample was found pure & complies with standard of IP.

Melting Point

The Melting point of Methylcobalamin IP was determined & it was found to be 133-155°C which complies with the standard.

Solubility Study

The solubility of Methylcobalamin IP has been determined, and is found to be sparingly soluble in water.

UV Spectrophotometric Analysis of Drug

The UV spectrum of Methylcobalamin IP was obtained in Methanol. The λ max of Methylcobalamin IP was found to be 356 nm. The UV spectrum of Methylcobalamin in Methanol is shown in figure 1 below.

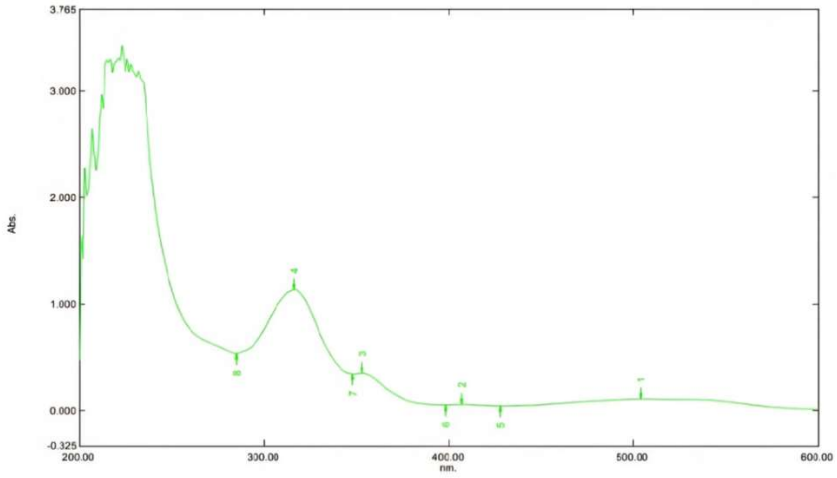


Figure 1. UV spectra of Methylcobalamin in Methanol at 356nm

Calibration curve of Methylcobalamin IP was prepared in Methanol. Absorbances at λmax 356 nm of various concentrations of Methylcobalamin in Methanol were measured. The calibration curve of Methylcobalamin IP in Methanol is as shown below in figure 2.

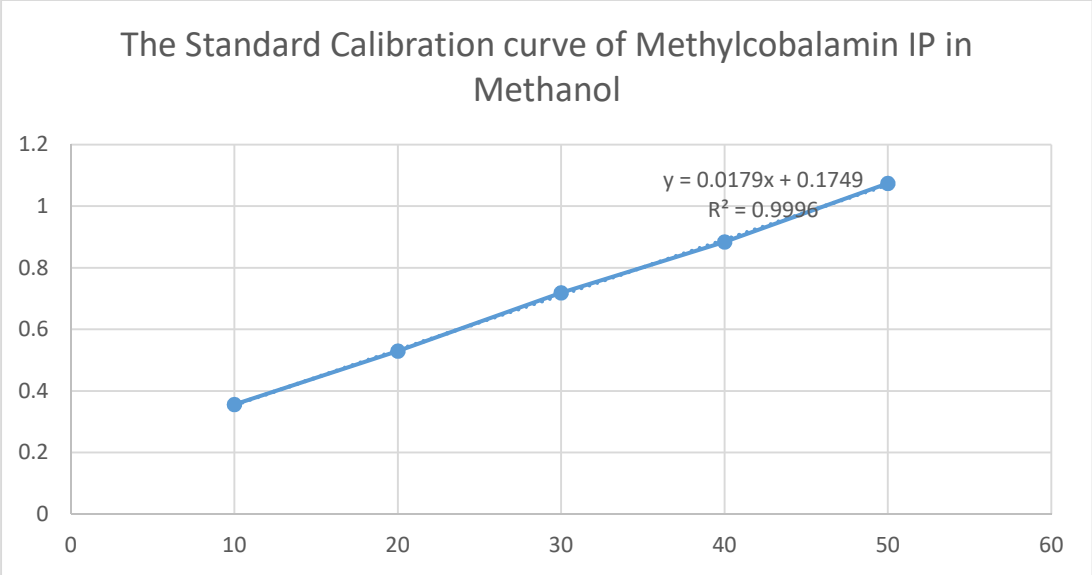
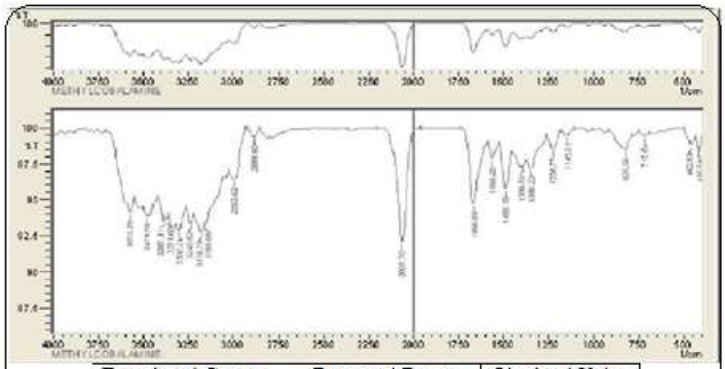


Figure 2. The Standard Calibration Curve of Methylcobalamin IP in Methanol

Drug Excipient Compatibility Study

FTIR Spectroscopy was conducted to check for any potential interaction that could occur between Methylcobalamin IP & the excipient used in the formulation.

FTIR of Methylcobalamin IP

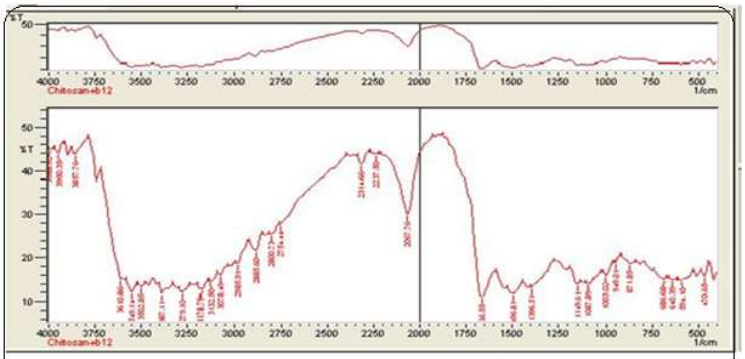


Functional Group	Reported Range	Obtained Value
O-H / N-H stretching	3200–3500	3302
C-H stretching	2850–3000	2885, 2992
Amide I (C=O)	1650–1680	1666
Amide II (N-H)	1550–1600	1566
Corrin Ring vibrations	1450–1500	1489
C-O stretching	1000–1250	1256

Figure 3. FTIR Studies of Methylcobalamin IP

The IR Spectra of the drug showed characteristic peaks which confirmed the structural integrity of the drug. Hence, there was no indication of impurity & the sample of Methylcobalamin IP was found pure (Figure 3).

FTIR of Methylcobalamin IP with Chitosan

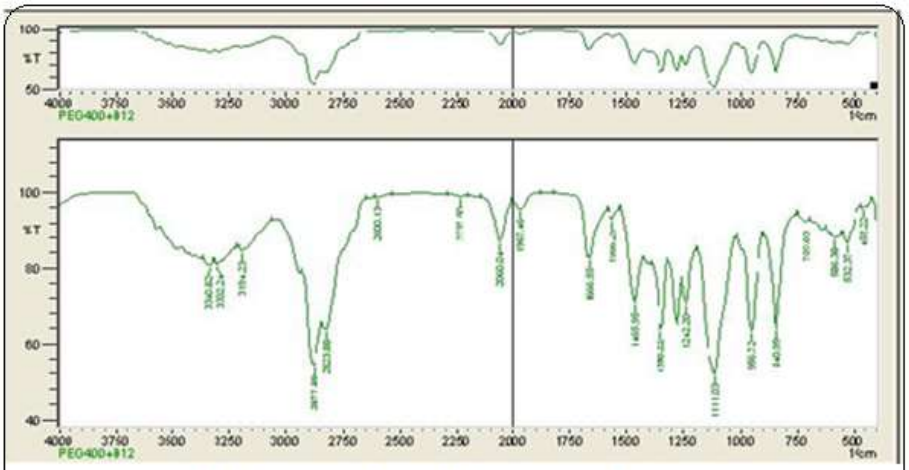


Functional Group	Reported Range	Obtained Value (cm- 1)
O-H / N-H stretching	3200–3500	3302
C-H stretching	2850–3000	2885, 2992
Amide I (C=O)	1650–1680	1656
Amide II (N- H)	1550–1600	1556
Corrin Ring	1450–1500	1454
C-O stretching	1000–1250	1077

Figure 4. FTIR Studies of Methylcobalamin IP with Chitosan

The FTIR Spectrum of the physical mixture of Methylcobalamin IP & Chitosan showed no significant change in major peaks of the drug, which suggested that there was no chemical reaction between Methylcobalamin IP & Chitosan (Figure 4). These validate that the drug will be compatible with the formulation components & support stability of the formulation.

FTIR of Methylcobalamin IP with PEG 4000



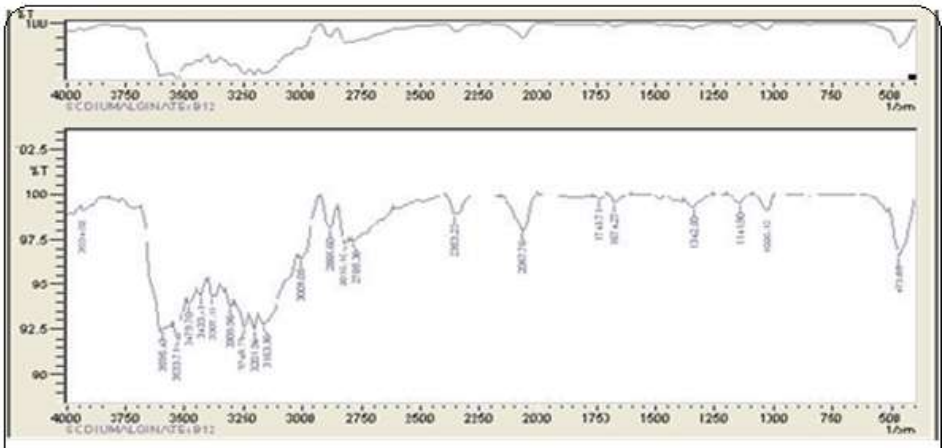
Functional Group	Reported Range (cm- 1)	Obtained Value (cm- 1)
O-H stretching	3200–3500	3340
C-H stretching	2850–3000	2877
Amide I (C=O)	1650–1680	1666
Amide II (N- H)	1550–1600	1566
Corrin Ring	1450–1500	1465
C-O stretching	1000–1250	1111

Figure 5. FTIR Studies of Methylcobalamin IP with PEG 4000

In the present case, no chemical reaction was observed between Methylcobalamin IP & PEG 4000 as there were

no significant changes in the major peaks (Figure 5). These assure compatibility of the drug with formulation constituents & stability of the final formulation.

FTIR of Methylcobalamin IP with Sodium Alginate



Functional Group	Reported Range (cm- 1)	Obtained Value (cm- 1)
O-H stretching	3200–3500	3307
C-H stretching	2850–3000	2920
C=O stretching (Carboxylate)	1600–1750	1674
C-O stretching	1000–1150	1020

Figure 6. FTIR Studies of Methylcobalamin IP with Sodium Alginate

The major peaks of the drug were retained without any significant changes were noticed, showing no chemical reaction between Methylcobalamin IP & Sodium Alginate (Figure 6). These are used to establish compatibility with the formulation components and to ensure that the final formulation is stable.

DSC of Methylcobalamin IP

The melting point of sample was determined by DSC analysis, which is one of the important thermal parameters and signifies the purity and crystalline nature of compound. The endothermic peak obtained in DSC thermogram was at 141.37 °C, which corresponds to the melting point of the substance as per IP (Figure 7).

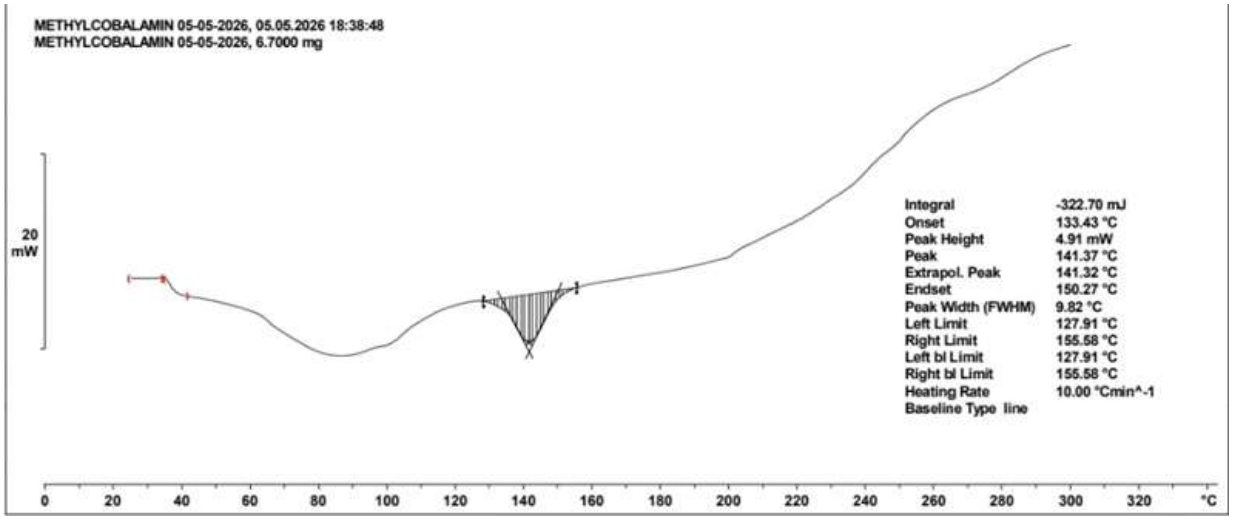


Figure 7. DSC Studies of Methylcobalamin IP

Occurrence of a single well-defined peak suggests sample is in pure & crystalline form with minimal impurities.

Preparation of Liposomes

Using the same method, a series of liposomal formulations (F1 to F8) were prepared by changing the concentration of the phospholipids, cholesterol and stirring speed as mentioned in table no10.8. The optimization was for particle size, zeta potential and entrapment efficiency in the batch. The best overall performance will be used as a guideline for further formulation of the gel. The formulation table of liposomes (F1 to F8) are given below (Table 2).

Table 2. Composition of liposomes

Ingredients	F1	F2	F3	F4	F5	F6	F7	F8
Methylcobalamin IP (mg)	5							
Soya Lecithin (mg)	100	200	100	200	100	200	100	200
Cholesterol (mg)	20	40	20	40	20	40	20	40
PBS (7.4 pH) (ml)	100							
Ethanol (ml)	10							
Stirring speed (rpm)	600	600	600	600	900	900	900	900
Sonication time (min)	10							
Sonication speed (rpm)	5000							

Evaluation of Liposomal Formulation of Liposomes

The prepared liposomal formulations (F1 – F8) were evaluated for a series of evaluation parameters to determine the physicochemical properties & performance. The evaluation comprised of particle size, zeta potential & entrapment efficiency. There are various batches and particle size (222.0nm to 358.2nm). The size of the particle was observed to decrease with the increasing speed of stirring and increase with increasing concentration of lecithin because more lipid material & more viscosity will decrease the size of the particle but cholesterol acts as an important bilayer stabilizer which will prevent clumping of particles. All the formulations exhibited the range of the values from (0.021 & 1.000) for the Polydispersity Index which indicate particle distribution. The Zeta Potential results of the different batches (F1 to F8) which range from (-9.40 to -23.6) contributes to the physical stability & low aggregation tendency of all the batches (Table 3).

Table 3. Characterisation of Liposomal formulation

Characterization	F1	F2	F3	F4	F5	F6	F7	F8

Particle size(nm)	338.6	266.0	222.0	358.2	283.1	254.2	245.6	264.5
PDI	0.850	0.251	0.162	1.000	0.259	0.225	0.166	0.021
Zeta Potential (Mv)	-9.40	-14.1	-20.7	-23.6	-21.9	-21.5	-17.9	-22.2

Particle size of Liposomal formulation Result

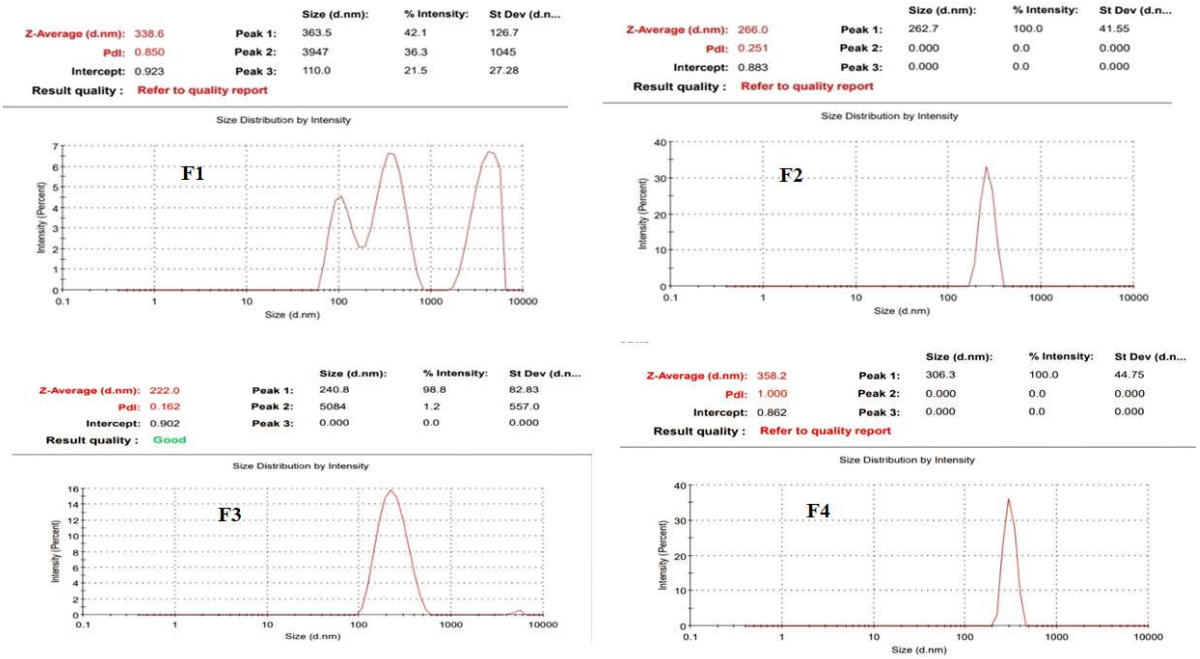


Figure 8. Particle size of Liposomal formulation F1-F4

Particle size analysis of the liposomal formulations showed mean vesicle size of 338.6 nm (F1), 266.0 nm (F2), 222.0 nm (F3) and 358.2 nm (F4) for the different batches, respectively (Figure 8).

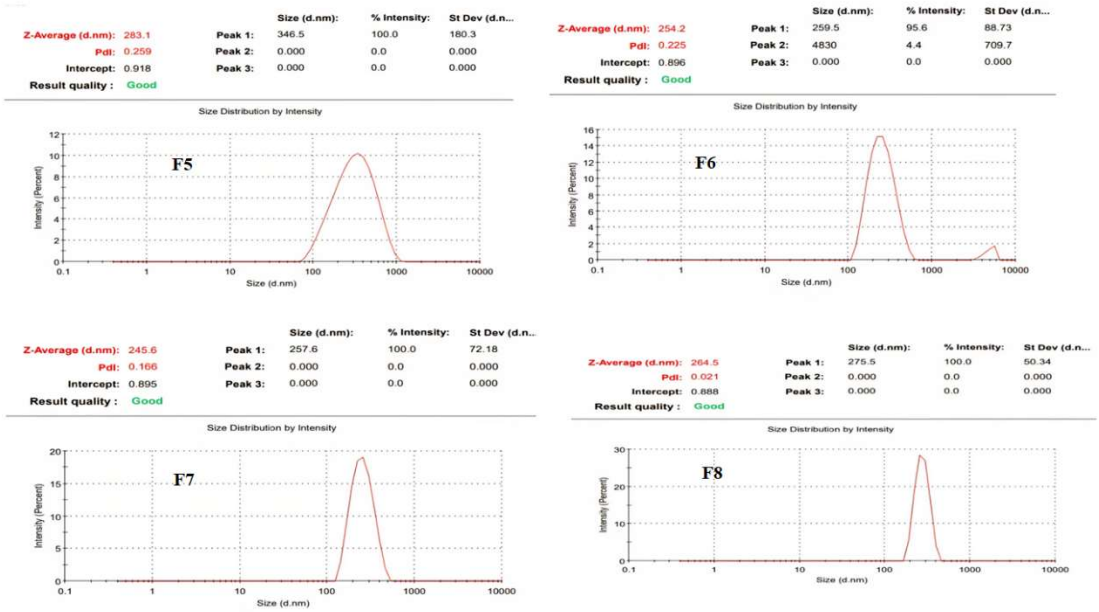


Figure 9. Particle size of Liposomal formulation F5-F8

The mean particle sizes of the liposomal formulations F5, F6, F7 and F8 were 283.1 nm, 254.2 nm, 245.6 nm and 264.5 nm, respectively, when visualized by particle size distribution graphs.

Zeta Potential of Liposomal Formulation Result

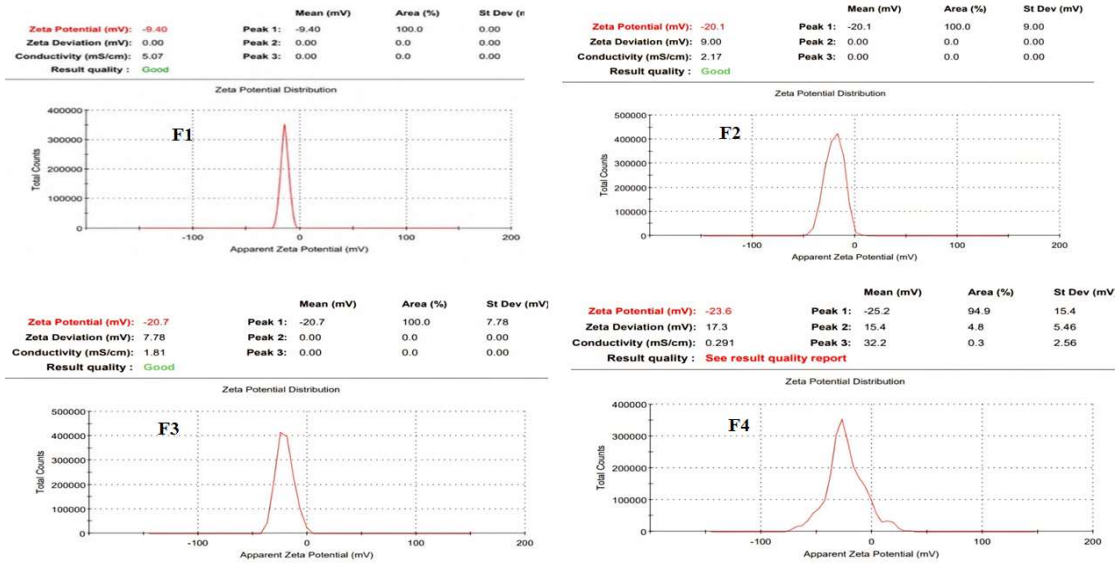


Figure 10. Zeta potential of Liposomal formulation F1-F4

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he zeta potential distribution plots of the liposomal formulations F1, F2, F3 and F4 were concluded to be −9.4 mV, −20.1 mV, −20.7 mV and −23.6 mV respectively.

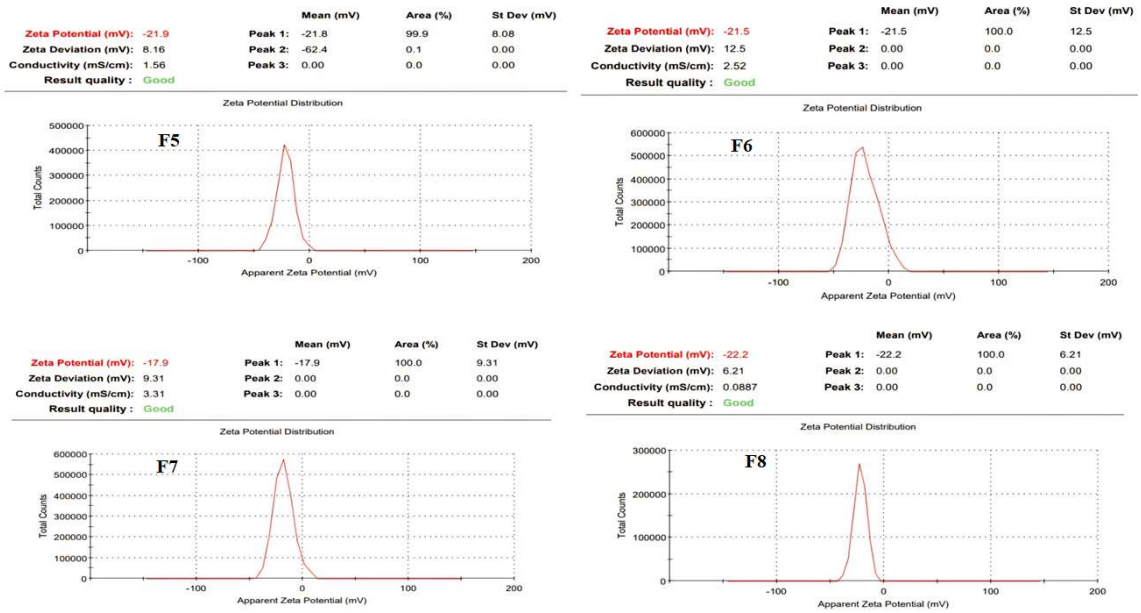

Figure 11. Zeta potential of Liposomal formulation F5-F8 batch

The zeta potential distribution curve of the liposomal formulations F5, F6, F7 and F8 were obtained at −21.9 mV, −21.5 mV, −17.9 mV, and +22.2 mV respectively showing a varying degree of colloidal stability for each formulation. Based on the above findings the F3 batch was chosen as optimized batch because of its smallest particle size 222 .0 nm, lower PDI 0 .162 and a good zeta potential -20 .7 mV which indicates that the batch has been optimized for a stable, effective & reproducible liposomal system.

Estimation of Entrapment Efficiency

One of the key specifications was the entrapment efficiency which indicated the ability of the formulation to deliver the product. To investigate the drug holding capacity of liposomes, the entrapment efficiency of all liposomal formulations (F1 – F8) was determined. The results indicated that there were differences between formulation, which were related mainly to the concentration of soya lecithin & cholesterol and the stirring speed. The entrapment efficiency % of F1 to F8 liposomal formulation was measured, the results were ranged from 68.53±1.41 to 93.24±1.15 (Figure 12). Formulation F3 gave the highest entrapment efficiency of 93.24±1.15(%) may be due to the optimum combination of soya lecithin, cholesterol & stirring speed which is found to stabilize the liposome structure and accommodate more drug into the liposome.

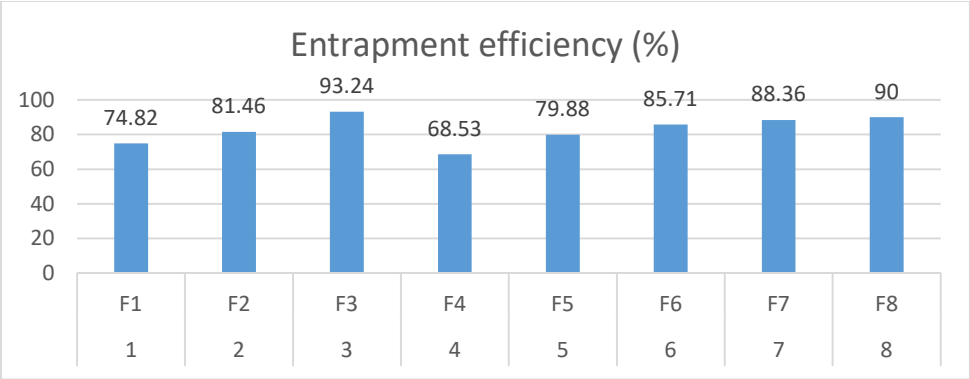

Figure 12. Entrapment efficiency of liposomes

Scanning Electron Microscopical Study

To observe the surface morphology & shape of optimized liposomal formulation F3, SEM was performed.

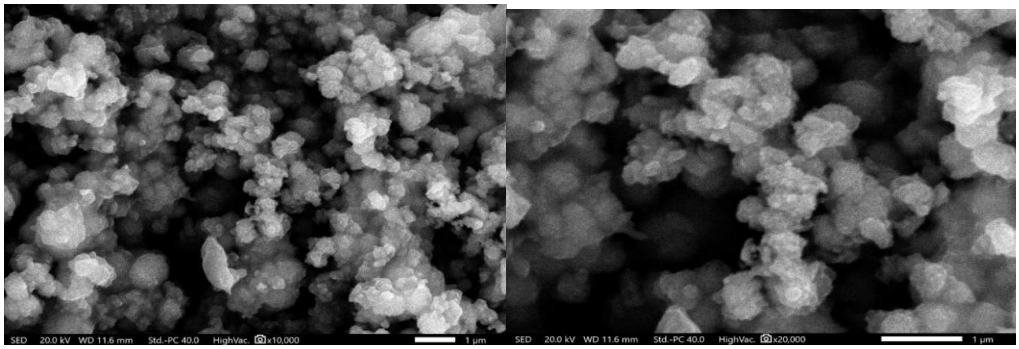

Figure 13. SEM Image of F3 Liposomes

The SEM morphology images indicated that the liposomes were spherical in shape and had smooth & uniform surface confirming successful formulation of liposomes. No significant aggregation and structural irregularities were observed which would suggest a stable formulation for further application.

Evaluation of Coated Liposomes

The prepared coated liposomal formulation of optimized batch F3 were subjected to series of evaluation parameter to assess their physicochemical properties & performance. The evaluation contained particle size, zeta potential, entrapment efficiency, SEM & in-vitro pH dependent dissolution studies. The coated liposomes showed the Entrapment efficiency of 96.78±0.88 %. The particle size after coating was increased from 222.0 nm to 298.3 nm and the PDI value was increased slightly from 0.162 to 0.251. This zeta potential value changed from −20.7 mV to -28.9 mV, showing effective deposition of coating material on the liposomal surface and enhanced colloidal stability (Table 4).

Table 4. Characterization of Liposomal formulation

Batch	**F3 Coated Liposomes**
Particle size(nm)	298.3 nm
PDI	0.251
Zeta Potential (mV)	28.9 mV

Particle size of Coated Liposomal Formulation

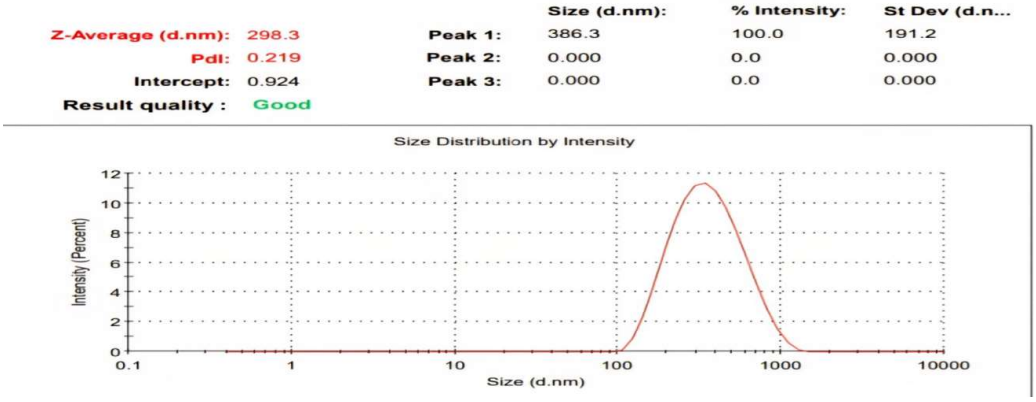

Figure 14. Particle Size of Coated Liposomal Formulation

The graph of Particle Size of Coated Liposomal formulation (Figure 14) shows particle size of 298.3 nm. The size of the liposomes increased from 222.0 nm to 298.3 nm and the PDI increased slightly from 0.162 to 0.251 after coating, representing successful deposition of chitosan coating, PEG 4000 & Sodium Alginate layers on liposomes.

Zeta Potential of Coated Liposomal Formulation

Surface morphology & shape of optimized liposomal formulation (F3) were studied using SEM.

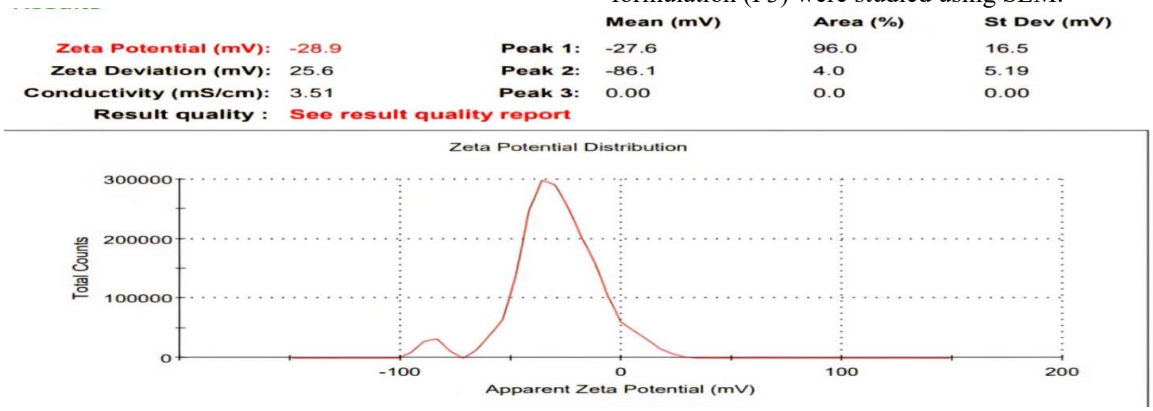

Figure 15. Zeta Potential of Coated Liposomal Formulation

The graph of particle size of coated liposomal formulation with -28.9 mV (Figure 15). The zeta potential value of formulation increased from-20.7 mV to 28.9Mv after coating indicating increase in the stability of the formulation after coating.

Entrapment Efficiency (%)

The entrapment efficiency of liposomal formulation after coating was found to be 96.78±0.88%. The entrapment efficiency increased from 93.24±1.15 to 96.78±0.88% which suggest that coating liposomes increased the entrapment efficiency of drug & thus stabilize the liposomes & accommodated more drug.

Scanning Electron Microscopical Study

Surface morphology & shape of the coated liposomal formulation of optimized batch F3 was done by SEM analysis (Figure 16). The SEM images revealed that the liposomes were spherical in shape with smooth & uniform surface confirming successful formulation of coated liposomes. No significant aggregation and structural irregularities were observed which would suggest a stable formulation that could be taken forward for further application.

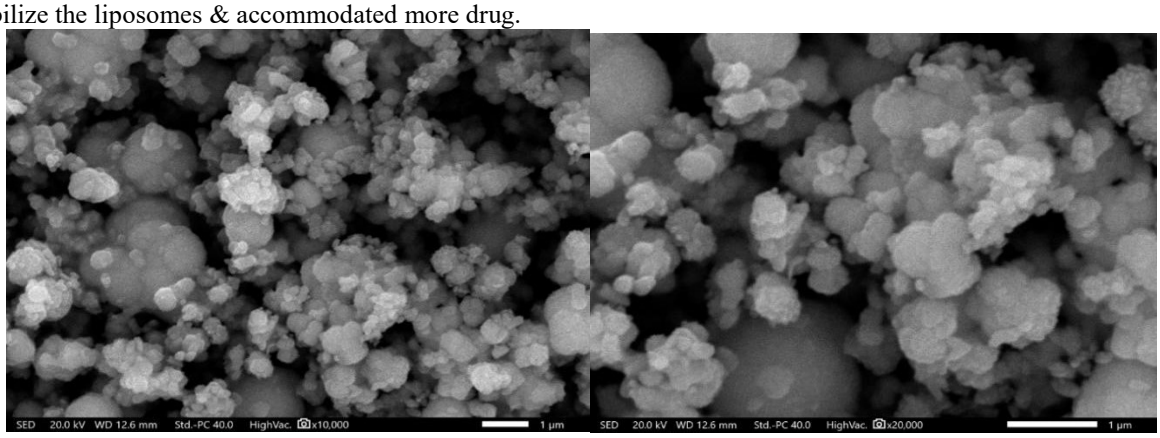

Figure 16. SEM Image of Coated Liposomes

***In-Vitro* Drug Release Study of Coated Liposomes at Various pH (1.2, 6.8, 7.4)**

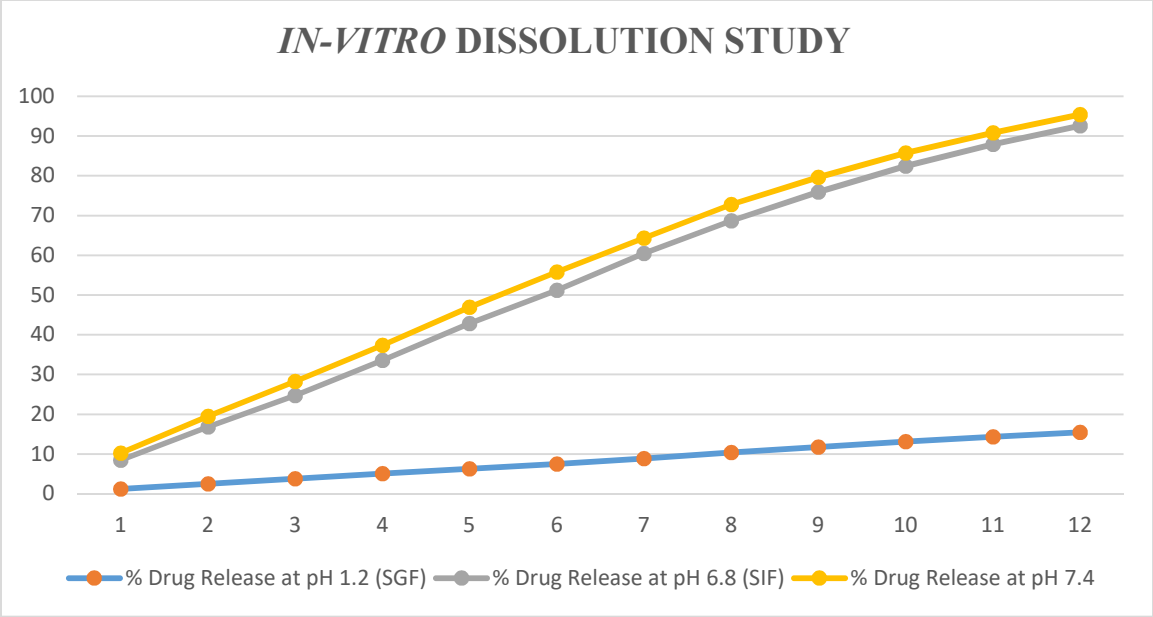

Figure 17. *In- Vitro* Dissolution Study

The dual coated enteric-coated chitosan-PEG liposomal drug release of methylcobalamin was investigated in the dissolution medium with pH1.2, pH6.8 and pH7.4 for 12 hours. The effect of pH on the release of the drug and the ability of the enteric coating on sustained protection in stomach environment and the promoting release of the drug in the intestine were evaluated. The formulation was found to release drugs in a pH dependent manner. During the research it was found that the formulation had a relatively low release of the medication in an acidic medium (pH 1.2). The drug release gradually increased from 1.2 ± 0.2% after 1 hour to 15.5 ± 0.7% after 12 hours (Figure 17). The low release at pH1.2 could be attributed to sodium alginate enteric coating, which prevented the drug release from being premature. The result shows that the formulation can be protective of the degradation of methylcobalamin in gastric environment. However, a much greater drug release was observed in simulated intestinal fluid (pH 6.8). The cumulative drug release increased from 8.5 ± 0.4% at 1 hour to 92.6 ± 1.1% after 12 hours. The improved release at pH 6.8 might be attributed to swelling and partial dissolution of enteric coating, which would help the dissolution medium to reach the liposomal vesicles and promote drug diffusion. At the same pH of 7.4, the formulation exhibited maximum drug release from all the media tested. The drug release was cumulative percentage drug release which increased from 10.2 ± 0.5% at 1 h to 95.4 ± 1.2% at 12 h. The higher release at pH 7.4 might be due to complete hydration and erosion of the polymeric coating which leads to effective release of methylcobalamin from the coated liposomes. Results show that developed formulation exhibits to be excellent pH-sensitive release formulation. A lack of drug release in acidic conditions and high drug release in intestinal pH represent good performance of enteric coating system. The coating with chitosan increased the stability of the liposomal vesicles and the coating with sodium alginate helped to control the release of the drug in gastric conditions. This release profile would be beneficial for oral administration of methylcobalamin since it would increase intestinal bioavailability and possibly increase intrinsic factor independent absorption of the drug. The optimized enteric-coated chitosan-PEG dual-coated liposomal formulation exhibited excellent gastric protection and controlled release properties along with high drug release at intestinal pH, which would make it an ideal carrier system for oral administration of methylcobalamin.

Comparative *In-Vitro* Drug Release Study of Marketed Formulation

The rapid dissolution observed in acidic medium suggests that there was no enteric barrier and thus a rapid release of methylcobalamin into the dissolution medium. The developed enteric coated chitosan-PEG dual coated liposomal formulation on the other hand, showed minimal release in pH 1.2 and enhanced release in intestinal pH, which shows that it has good pH responsive behavior.

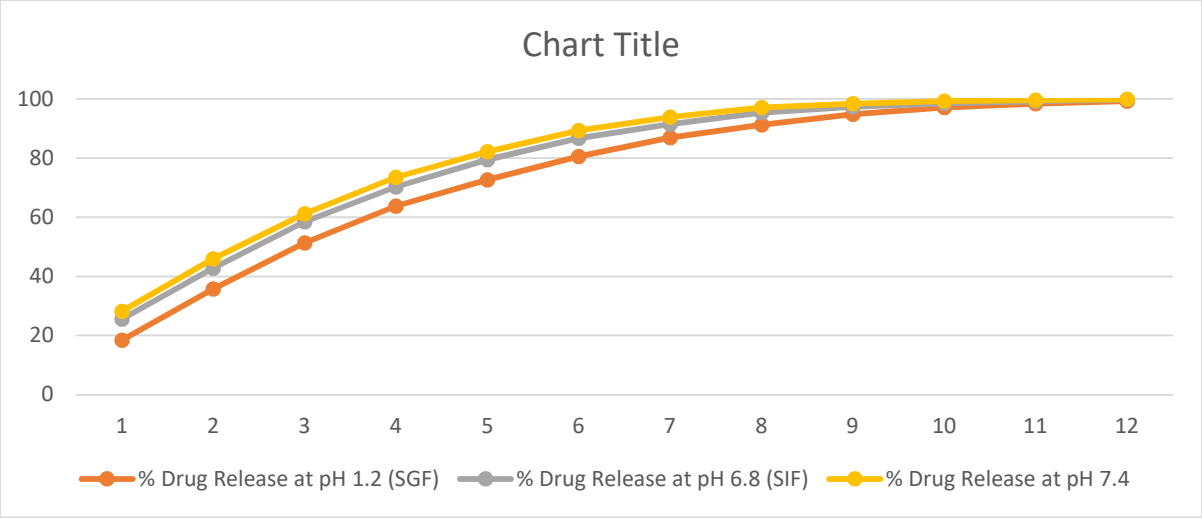

Figure 18. *In-Vitro* Dissolution Study of marketed formulation

As evident from the comparison, the marketed formulation is an immediate release dosage form, while the newly developed liposomal formulation is gastric protecting & controlled drug release. This controlled release property could enhance intestinal bioavailability, and provide intrinsic factor-independent oral bioavailability of methylcobalamin.

Stability Study of Coated Liposomal Formulation:

The stability studies of the coated formulation (F3) were carried out at storage conditions (4°C) over a period of 3 months (Table 5). The formulation was tested after 3 months for the changes in particle size, EE% & Zeta Potential.

Table 5. Characterization of coated liposomes after 3 months

Parameter	Storage condition	Initial results	Results after 3 months
EE%	4°C	96.78±0.88%	94.47±0.75
Particle size	4°C	298.3	299.6
Zeta potential	4°C	-28.9	-26.6

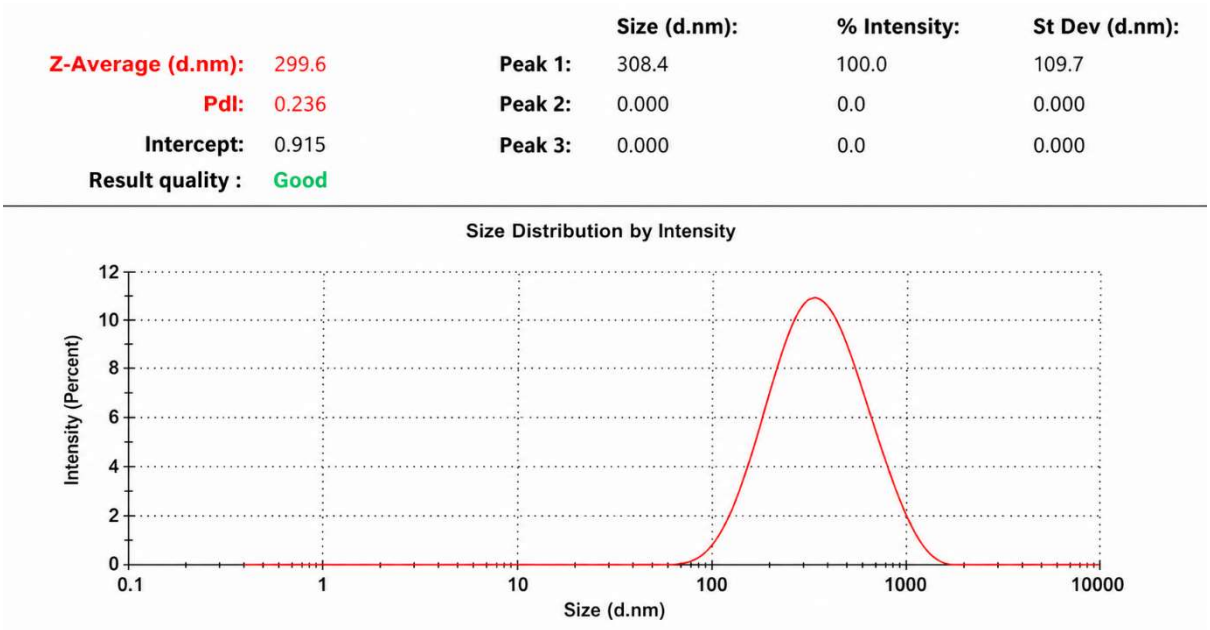

Figure 19. Particle size of Coated Liposomes after 3 months

The PS of coated liposomes after 3 months is displayed in the graph (Figure 19), which indicates particle size of 299.6. The result revealed that there was no significant difference in the value of the zeta potential which showed that formulation was physically & chemically stable (Figure 20).

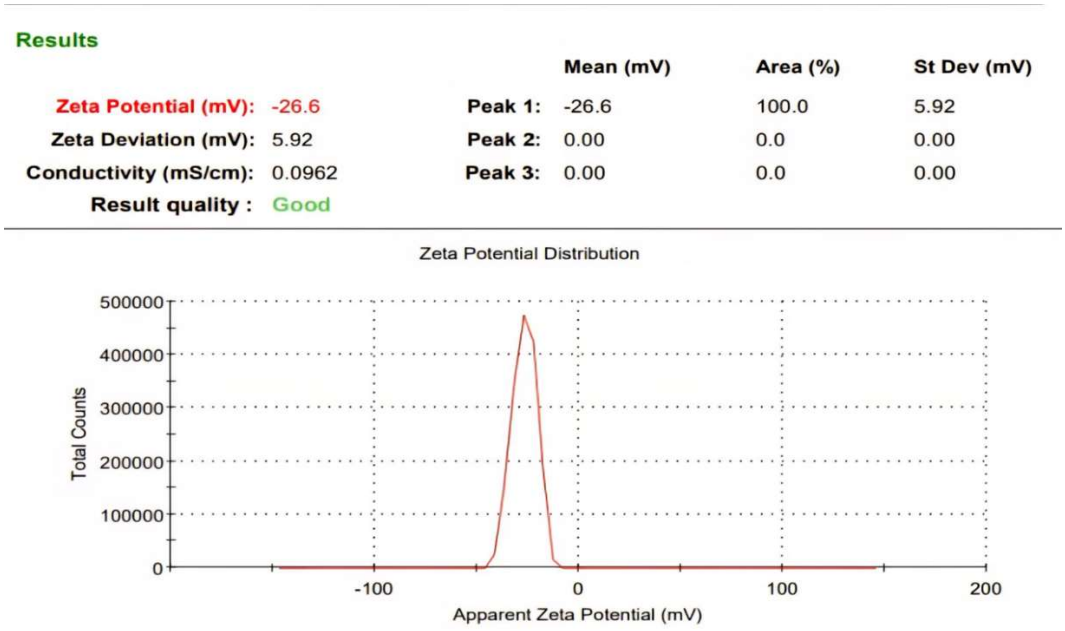

Figure 20. Zeta Potential of coated Liposomes after 3 months

CONCLUSION

The present study has successfully shown the feasibility of multilayered lipidosome to enhance oral delivery of Vitamin B12. The liposomal encapsulation of the vitamin B12, chitosan coating, PEG 4000 functionalization and enterically protected by sodium alginate appears to be a complete solution to surmount the physiological and formulation related barrier to the vitamin B12 absorption. The developed system provides increased protection against gastric degradation, increased mucoadhesion, increased intestinal permeation, prolonged gastrointestinal residence time and controlled release in the intestinal environment. The combination of chitosan and PEG 4000 provides both the interaction with mucus and penetration, and an alginate provides site-specific release of intact liposomes in the small intestine. The formulation would also be expected to greatly enhance the oral bioavailability as it would use both the transcellular and the paracellular transport pathway, thus avoiding the intrinsic factor pathway. Thus, the new oral nanocarrier strategy could be a viable alternative to the current vitamin B12 supplementation strategy. The novel delivery strategy developed could represent a broad platform for the oral delivery of other weakly absorbed vitamins, peptides, proteins and hydrophilic drug candidates in addition to meeting an important clinical need for patients with a compromised intrinsic factor function.

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