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EFFECT OF LECITHIN AND CHOLESTEROL CONCENTRATIONS ON SIMVASTATIN LIPOSOMES

Maheswaran A*, Ajay kumar R, Anisha KS, Anusha K.

Department of Pharmaceutics, Jaya College of Paramedical Sciences, College of Pharmacy, Thirunindravur, Chennai, Tamilnadu, India.

***Corresponding Author**

Prof.A.Maheswaran,
Principal, Jaya College of Paramedical Sciences, College of Pharmacy,
Thirunindravur, Chennai, Tamilnadu, India.
maheswaranphd2013@gmail.com.

Abstract

The current study reveals the effect of Lecithin and Cholesterol concentrations on simvastatin liposomes. Simvastatin is a HMG Co-A reductase inhibitor class drug, used in treatment of lowering lipid levels. The Simvastatin Liposomes were prepared by Thin Film Hydration technique with various concentrations of Lecithin and Cholesterol. The Pre-formulation studies are done by UV spectrophotometer and DSC study which shows that there is no interaction between the drug and excipients. The prepared Liposomal formulations of various concentration were evaluated by Drug content, particle size, Zeta potential, entrapment efficiency, SEM analysis and *In-vitro* drug release study were studied. From this study, the prepared Liposomal formulation of various concentrations reveals the best formulation which is chosen as newly formulated controlled liposomal drug delivery system.

Keywords: *Simvastatin liposomes, entrapment efficiency, drug content, cholesterol lowering effects.*

Introduction

Liposomes are small artificial vesicles of spherical shape that can be created from cholesterol and natural nontoxic phospholipids. Due to their size and hydrophobic and hydrophilic character (besides biocompatibility), liposomes are promising systems for drug delivery. Liposome properties differ considerably with lipid composition, surface charge, size and the method of preparation. Furthermore, the choice of bilayer components determines the 'rigidity' or 'fluidity' and the charge of the bilayer.

For instance, unsaturated phosphatidylcholine species from natural sources (egg or soybean phosphatidylcholine) give much more permeable and less stable bilayers, whereas the saturated phospholipids with long acyl chains (for example, dipalmitoyl phosphatidylcholine) form a rigid, rather impermeable bilayer structure[1-3].It has been displayed that phospholipids impulsively form closed structures when they are hydrated in aqueous solutions. Such vesicles which have one or more phospholipid bilayer membranes can transport aqueous or lipid drugs, depending on the nature of those drugs. Because lipids are amphipathic (both hydrophobic and hydrophilic) in aqueous media, their thermodynamic phase properties and self-assembling characteristics influence entropically focused confiscation of their hydrophobic sections into spherical bilayers. Those layers are referred to as lamellae [4].

Liposomes are extensively used as carriers for numerous molecules in cosmetic and pharmaceutical industries. Additionally, food and farming industries have extensively studied the use of liposome encapsulation to grow delivery systems that can entrap unstable compounds (for example, antimicrobials, antioxidants, flavours and bioactive elements) and shield their functionality [5-7].

Liposomes can trap both hydrophobic and hydrophilic compounds, avoid decomposition of the entrapped combinations and release the entrapped at designated targets. Because of their biocompatibility, biodegradability, low toxicity and aptitude to trap both hydrophilic and lipophilic drugs [8] and simplify site-specific drug delivery to tumour tissues [9], liposomes have increased rate both as an investigational system and commercially as a drug delivery system. Many

studies have been conducted on liposomes with the goal of decreasing drug toxicity and/ or targeting specific cells [10].

Liposomal encapsulation technology (LET) is the newest delivery technique used by medical investigators to transmit drugs that act as curative promoters to the assured body organs. This form of delivery system proposal targeted the delivery of vital combinations to the body. LET is a method of generating sub-microscopic foams called liposomes, which encapsulate numerous materials.

These 'liposomes' form a barrier around their contents, which is resistant to enzymes in the mouth and stomach, alkaline solutions, digestive juices, bile salts and intestinal flora that are generated in the human body, as well as free radicals. The contents of the liposomes are therefore, protected from oxidation and degradation [11].

Liposomes were evaluated by optical microscope as well as transmission electron microscope. Analysis of drug content was carried on UV spectrophotometer. *In vitro* release rate studies were conducted on specially designed *In vitro* model and *in vivo* studies of liposomes were performed on albino rabbit eyes [12].

2. MATERIALS

Various materials used in the formulation are as follows

Table 2.1. Materials used

S.NO	Materials	Supplier
1	Simvastatin	Sigma Aldrich pvt.ltd
2	Lecithin	Sigma Aldrich pvt.ltd
3	Cholesterol	Sigma Aldrich pvt.ltd
4	Mannitol	Rankem
5	Chloroform	Rankem
6	Methanol	Rankem
7	Potassium di hydrogen phosphate	Rankem
8	Ortho phosphoric acid	Rankem

List of Equipment:

Table 2.2. Equipment used

S.NO	EQUIPMENTS	MODE
1.	Electronic balance	Mettler Toledo AG 135.
2.	Ultra-centrifuge	Remi instruments, Mumbai.
3.	Mechanical stirrer	Remi instrument.
4.	DSC	Shimadzu DSC-60.
5.	Particle size analyser	Malvern master sizer.
6.	UV spectrophotometer	Shimadzu 1710, Mumbai.
7.	USP dissolution apparatus	Lab India, DS8000.

3. METHODS:

PREFORMULATION STUDIES:

Development of Calibration curves:

Preparation of Hydrochloric acid buffer pH 1.2:

8.9 ml of concentrated HCl was diluted in 1000 ml volumetric flask with distilled water.

Preparation of Phosphate buffer pH 6.8:

28.80 gm of disodium hydrogen phosphate and 11.45 gm of potassium dihydrogen phosphate was weighed and dissolved in 1000ml volumetric flask with distilled water.

Preparation of Phosphate buffer pH 7.4:

8 gm of sodium hydroxide pellets and 27.212 gm of Potassium dihydrogen phosphate was weighed and dissolved in 1000 ml volumetric flask with distilled water.

Linear plot of Simvastatin in pH 1.2 buffer:

100 mg of Simvastatin was dissolved in pH 1.2 buffer in a 100 ml standard flask, which gives 1mg/ml. From this stock solution suitable dilutions were made with pH 1.2 buffer in order to get 10 µg-50 µg/ml concentrations. Absorbance of these solutions were measured at 260 nm using UV-visible spectrophotometer and standard graph was plotted.

Linear plot of Simvastatin in pH 6.8 phosphate buffer:

100 mg of Simvastatin was dissolved in 6.8 buffer in a 100 ml standard flask, which gives 1 mg/ml. From this stock solution suitable dilutions were made with 6.8 in order to get 10 µg-50 µg/ml concentrations. Absorbance of these solutions were measured at 260 nm using UV- visible spectrophotometer and standard graph was plotted.

Linear plot of Simvastatin in pH 7.4 phosphate buffer:

100mg of Simvastatin was dissolved in 7.4 buffer in a 100ml standard flask, which gives 1mg/ml. From this stock solution suitable dilutions were made with 7.4 buffer in order to get 10µg-50µg/ml concentrations. Absorbance of these solutions were measured at 260 nm using UV- visible spectrophotometer and standard graph was plotted.

Drug-excipient compatibility study by DSC:

Differential scanning calorimetry (DSC):

Samples of individual components as well as each drug-excipient were weighed (Mettler Electronic balance) directly in pierced aluminum crucible pans (5-10 mg) and scanned in the 50-300°C temperature range under static air, with heating rate of 10 ºC /min, using Shimadzu DSC-60 equipment [13].

PREPARATION OF SIMVASTATIN LIPOSOMES BY THIN FILM HYDRATION TECHNIQUE:

Simvastatin liposomes were prepared by thin film hydration technique. Liposomal formulations were prepared by dissolving Lecithin, Cholesterol in various concentrations and drug in approximately 50 ml of chloroform. The solution was then taken in 500 ml round bottom flask. Chloroform was evaporated under vacuum 100 mmHg using rotary evaporator at 63 ºC to form a thin film. Evaporation was continued for approximately 15 min until dry residue is formed. The organic solvent is eliminated slowly by this method to yield a thin lipid film on the interior surface of the flask. To ensure complete evaporation of the organic solvent, the films were vacuum dried for overnight. The film was than hydrated with different amount of phosphate buffer (pH 7.4) solution containing 1g of mannitol and rotated for 45 minutes. The liposomal suspension was kept overnight to get complete lipid hydration at 4 ºC. The formula used for the preparation of Simvastatin Liposomes were given in Table 3.1[14,15].

Table 3.1. Formula used for the preparation of Simvastatin Liposomes

S.NO	FORMULATION	DRUG (mg)	Lecithin (mg)	Cholesterol (mg)
1.	SLF1	10	100	25
2.	SLF2	10	125	50
3.	SLF3	10	150	75
4.	SLF4	10	175	100
5.	SLF5	10	200	125

CHARACTERIZATION STUDIES: [16-21]

- ✓ Particle size
- ✓ Zeta potential
- ✓ Drug content
- ✓ Entrapment efficiency
- ✓ SEM analysis
- ✓ *In vitro* drug release study

Particle size and Zeta potential (surface charge):

Surface charge is important in adhesion and interaction of particle with cells. The zeta- potential is used to measure the cell surface charge density. It can be measured using Malvern-Zetasizer. The prepared Simvastatin Liposomes were evaluated for their particle size and surface charge by photon correlation spectroscopy (PCS) using zetasizer.

The formulations were diluted to 1:1000 with the aqueous phase of the formulation to get a suitable kilo counts per second (kcps). Analysis was carried out at 25°C with an angle of detection of 90°. In this experiment six replicates were taken for the measurement. The results were given in results and discussion section.

Drug Content:

500mg of Simvastatin Liposomes were accurately weighed and transferred into a 25ml volumetric standard flask. The sample was dissolved with methanol, 1ml of this solution was diluted to 25ml with the purified water. The standard Simvastatin was dissolved and diluted with same methanol and water

respectively. Then the standard and sample absorbance was measured at 260 nm using UV-Visible spectrophotometer. The percentage of drug content was calculated.

Entrapment Efficiency:

The Simvastatin Liposomes in buffer solutions were subjected to centrifugation at 15000 rpm for 30 min. The supernatant liquid was separated and 1ml of this solution was diluted with buffer solution and the absorbance was measured at 260nm.

The amount of Simvastatin un entrapped in the supernatant was calculated. The amount of Simvastatin entrapped was determined by subtracting amount of free unentrapped Simvastatin from the total amount of Simvastatin taken for the preparation.

Scanning Electron Microscopy (SEM):

For the external morphology studies, air dried particles were visualized using scanning electron microscopy (FEI-Quanta 200F) operating at 15 kV. The samples were mounted on a metal slab with double adhesive tape and coated with platinum under vacuum.

***In Vitro* Drug Release Study:**

In vitro release studies were performed for 5 h using dialysis membrane by using the Franz diffusion cell. The prepared Simvastatin liposomes formulations equivalent to 10mg of Simvastatin (SLF1-SLF5) were placed inside a dialysis membrane and immersed in buffer pH 6.8.

At predetermined time intervals the sample was withdrawn and the amount of Simvastatin released was determined by measuring the absorbance at 260nm using a UV-Visible spectrophotometer. From the absorbance values the cumulative percentage drug release was calculated.

4. RESULTS AND DISCUSSION

Pre-formulation studies:

Preparation of Calibration graph for Simvastatin:

Standard calibration data of Simvastatin in pH 1.2, 7.4 and 6.8 buffers at 260nm

Table 4.1. Absorbance of Simvastatin in buffer solutions

S. No	Concentration (µg/ml)	Absorbance		
		pH 1.2	**pH 7.4**	**pH 6.8**
1	**0**	**0**	**0**	**0**
2	**10**	0.025	0.062	0.105
3	**20**	0.051	0.125	0.211
4	**30**	0.076	0.188	0.317
5	**40**	0.101	0.248	0.423
6	**50**	0.126	0.311	0.529

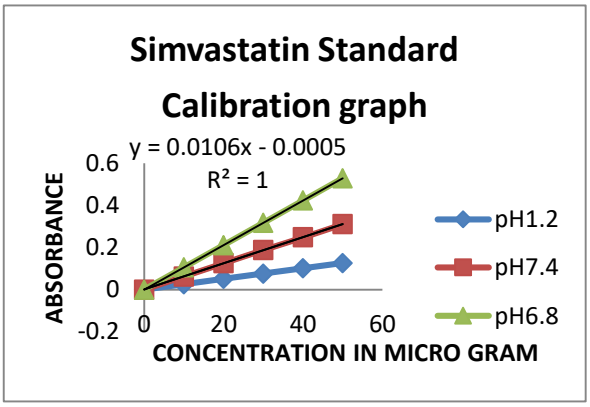

Fig. 4.1. Calibration curve of Simvastatin in pH 1.2, 7.4 and 6.8 buffers

Standard calibration curve of Simvastatin was carried out in 1.2 pH, 7.4 pH and 6.8 pH buffer at 260nm. The r^2 value in the entire medium shows nearly 1, which signifies linearity.

Drug - excipient Compatibility study using DSC:

DSC of Simvastatin showed a sharp endothermic peak at 134.11^{0}C (melting point). The physical mixture of Simvastatin and other excipients also showed the same thermal behavior (136.02^{0}C) as the individual component. DSC results also revealed that the physical mixture of Simvastatin with excipients showed superimposition of the thermograms. There was no significant change observed in melting endotherm of physical mixture of Simvastatin and excipients.

From the DSC studies, it was found that there were no interaction took place between Simvastatin and the other ingredients used in the formulation of press coated tablets. The DSC images were shown from fig 4.2 and 4.3

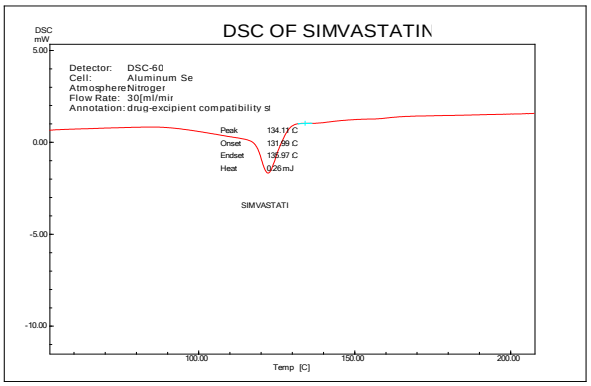

Fig 4.2

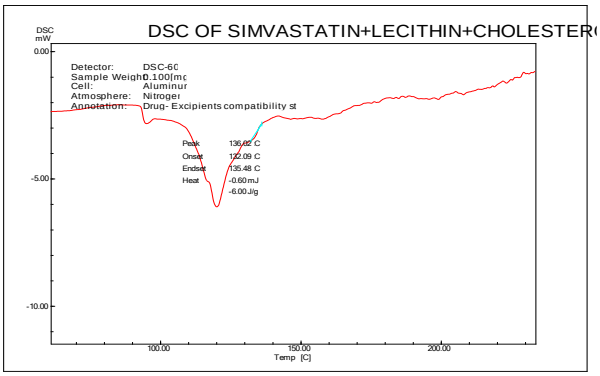

Fig 4.3

Fig.4.2 and 4.3. DSC Thermogram of Simvastatin and Simvastatin liposomes

Drug –Excipients accelerated compatibility study - Physical observation and assay:

Upon analysis of the drug excipient mixture for their physical characteristics no colour change was observed. Based on the chemical evaluation it was found that there was no significant change observed indicating that the drug is compatible with the added ingredients. The results of this study were given in Table 4.2- 4.5

Physical characteristics:

Physical characteristics results of Simvastatin indicated that, it possess poor flow property and needs to be improved. The results were given below (Table 4.2)

Table 4.2 Physical characteristics of individual drug and excipients:

S. No	Physical parameters	Results
1	Description	White crystalline powder
2	Melting point	134-137^{0}C
3	Loss on drying	0.02%
4	Assay	99.75%

Table 4.3 Physical characteristics of individual drug and excipients:

S. No	Sample ID	Initial description	Final description
1.	Simvastatin	White crystalline powder	No change
2.	Lecithin	Brown coloured semisolid mass	No change
3.	Cholesterol	White coloured powder	No change

Table 4.4 Physical characteristics of drug-excipient mixture:

S. No	Sample ID	Initial description	Final description
1	Simvastatin	White crystalline powder	No change
2	Simvastatin+Lecithin	Yellowish powder	No change
3	Simvastatin+Cholesterol	White coloured powder	No change

Table 4.5 Chemical characteristics of drug-excipient mixture:

S. No	Sample ID	Initial assay (%)	Final assay (%)
1.	Simvastatin	99.75	99.74
2.	Simvastatin + Lecithin	99.75	99.74
3.	Simvastatin+Cholesterol	99.75	99.74

Effect of Lecithin and Cholesterol Concentrations on Particle size, zeta potential, drug content and entrapment efficiency of Simvastatin Liposomes:

Table.4.6 Particle size, zeta potential, Drug content and entrapment efficiency of Simvastatin Liposomes:

Trials	Particle size (nm)	Zeta potential (mV)	Drug Content (%)	Entrapment Efficiency (%)
SLF1	271.8	-21.8	99.75	41.67
SLF2	273.5	-22.6	99.74	52.81
SLF3	277.4	-23.5	99.75	61.76
SLF4	278.6	-24.8	99.74	72.72
SLF5	**280.2**	**-26.4**	**99.75**	**85.88**

Particle size and entrapment efficiency of the Simvastatin Liposomes (**SLF1- SLF5**) were increased with increasing Lecithin and Cholesterol concentration. The entrapment efficiency of Simvastatin Liposomes (**SLF1- SLF5**) was found to be influenced by amount of Lecithin and Cholesterol used in the formulation of liposomes. This may be due to the fact that increase in Cholesterol concentration in the Simvastatin Liposomal formulations increases the rigidity of lipid bilayer of liposomes which in turn causes more stability and decreased permeability of the liposomal membrane leading to high entrapment efficiency and therefore increase in Lecithin concentration leads to formation of larger aqueous core volume of Simvastatin liposomes, which can entrap more amount of Simvastatin. **(SLF5)**.Increase in the zeta potential values were observed with increase in Lecithin and Cholesterol concentrations which increases the stability of prepared liposomes. Based on the results of Particle size, zeta potential, Drug content and entrapment efficiency of the prepared Simvastatin Liposomes (**SLF1- SLF5**), the trial **SLF5** which contains **200mg of Lecithin** and **125mg Cholesterol** concentrations was selected as the best formulation.

SEM ANALYSIS:

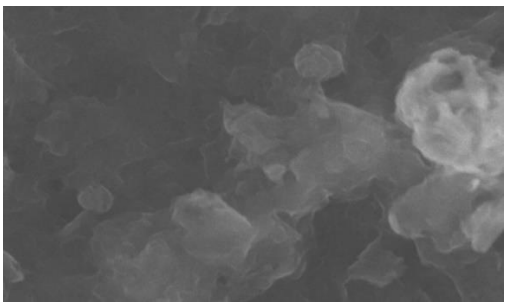

Fig.4.4. SEM ANALYSIS OF SIMVASTATIN LIPOSOMES- SLF5

The results of SEM analysis confirm the prepared liposomes were spherical in shape and nano in size. The SEM images of liposomes prepared with lecithin and Cholesterol were observed as well-identified perfect spheres and exist in disperse as well as aggregated collection. In all the formulations, the liposomes were spherical shaped vesicles.

The aggregation of liposomes was observed with lower Lecithin and Cholesterol concentrations **(SLF1)** whereas on increasing Lecithin and Cholesterol concentrations **(SLF5)** the reduced the aggregation of liposomes were observed. The results of SEM analysis suggested that Lecithin and Cholesterol is a stabilizing agent in the preparation of Simvastatin liposomes and may reduce the aggregation of liposomes.

***In- vitro* drug release**

Table 4.7 *In vitro* release studies of Simvastatin liposomes

S.NO	Time (mts)	%CUMULATIVE DRUG RELEASE				
		SLF1	SLF2	SLF3	SLF4	SLF5
1	**0**	**0**	**0**	**0**	**0**	**0**
2	30	**99.74**	**70.67**	**62.86**	**52.82**	**41.32**
3	60	99.74	**99.74**	**85.24**	**61.35**	**52.48**
4	120	99.75	99.74	**99.75**	**73.71**	**60.91**
5	180	99.75	99.74	99.74	**86.77**	**75.67**
6	240	99.74	99.74	99.74	**99.75**	**83.55**
7	300	99.75	99.74	99.74	99.74	**99.75**

In-vitro release data expressed as cumulative percentage release of Simvastatin over 300mts were determined for the Simvastatin liposomes containing varying Lecithin and Cholesterol concentrations were shown in Table 4.7.

From the *in vitro* drug release study results, the maximum percentage drug release 99.75% at the end of 300mts was observed with trial **SLF5** which contains **200mg of Lecithin** and **125mg of Cholesterol.**

Simvastatin Liposomes **SLF1** containing lower concentration of **Cholesterol (25mg)** and **Lecithin (100mg)** exhibited the fastest release 99.75% within 30mts among all liposomal formulations

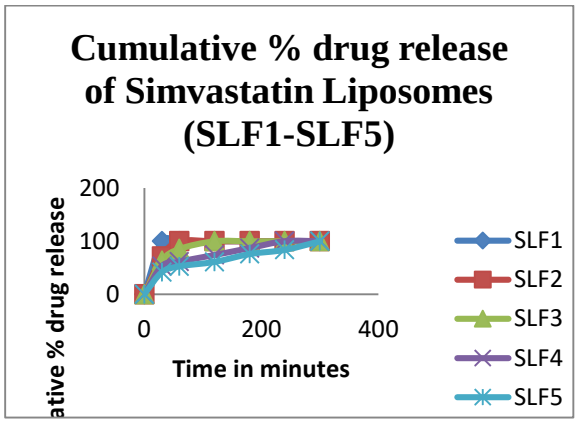

Fig.4.5

Below 200mg of Lecithin and 125mg of Cholesterol concentration as in the case of trials **SLF1-SLF4**, the drug release was very fast and the maximum percentage drug release 99.75% were obtained at the end of 30.60,120 and 240mts for the trials SLF1, SLF2, SLF3, and SLF4 respectively which was not desirable.

On increasing Lecithin concentration from **100mg-200mg** and Cholesterol concentration from **25mg-125mg**, the reduction in drug release was observed for all the trials **SLF1-SLF4**. The maximum percentage drug release for **SLF5** was found to be 99.75% at the end of 300mts was obtained.

Discussion

The release profiles of Simvastatin liposomes were biphasic, showing a relatively large burst release over the first hour due to drug detachment from liposomal surface, followed by a slower release phase. The burst release varies with the liposomal composition which is dependent on the concentration of drug, Cholesterol and Lecithin used in the formulation of liposomes.

The concentration of Lecithin and Cholesterol present in Simvastatin liposomal formulations had significant effect on *in-vitro* drug release of Simvastatin.

The in-vitro drug release results of Simvastatin liposomes confirmed the extension of Simvastatin release with increasing Lecithin and Cholesterol concentrations **(SLF1-SLF5)** in the Simvastatin liposomal formulations

This may be due to Cholesterol has cementing role in Simvastatin liposomal formulation which improves the integrity of the liposomal vesicles, also the presence of cholesterol in the lipid bilayer above phase transition temperature of Lecithin, alters the membrane fluidity by limiting the movement of relatively mobile hydrocarbon chains, reducing membrane permeability and decreasing the release of the entrapped Simvastatin, resulting in the retention of Simvastatin.

From the above evaluation results of all the Simvastatin liposomal formulations **(SLF1-SLF5)**, **SLF5** was selected as best formulation due to its ideal **particle size (280.2 nm)**, **Zeta Potential (-26.4), high entrapment efficiency (85.88%)** and desirable **drug release 99.75 %** at the end of 300mts.

5. SUMMARY AND CONCLUSION

In the present study liposomes containing Simvastatin was prepared. The active pharmaceutical ingredient Simvastatin was evaluated for its physical and chemical properties. The results obtained were satisfactory.

Simvastatin liposomes were prepared by thin film hydration technique and the Lecithin and Cholesterol concentrations were optimized by various trials. The effect of increase in Lecithin and Cholesterol concentration in various parameters like particle size, zeta potential, drug content, entrapment efficiency SEM Analysis and *in vitro* release profile were studied. Based on evaluation results of all the Simvastatin liposomal formulations **(SLF1-SLF5)**, **SLF5** was selected as best formulation due to its ideal **Particle size (280.2 nm)**, **Zeta Potential (-26.4mV), Entrapment efficiency (85.88%)** and **Drug release 99.75 %** at the end of 300mts and it was found to be suitable formulation to control the increased blood Cholesterol level. Hence it can be concluded that the newly formulated controlled release liposomal drug delivery systems of Simvastatin may be ideal and effective in the treatment of Hypercholesterolemia.

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