

Development and Characterization of Solid-Lipid Microparticles of Highly Insoluble Drug Sirolimus

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Abstract

Solid lipid microparticles (SLMs) represent an alternative carrier system to traditional colloidal carrier such as emulsion, liposomes and polymeric micro and nanoparticles. The purpose of this research work was to develop and evaluate solid lipid microparticles of sirolimus for oral delivery. Sirolimus is an immunosuppressive drug used to prevent transplant rejection and to treat auto immune diseases. It is a macrolide lactone produced by *streptomyces hygroscopicus*. It is extremely hydrophobic. Sirolimus solid lipid microparticles were prepared by hot homogenization technique. The matrix chiefly consisted of glyceryl monostearate and sodium taurocholate. The SLMs were studied for its particle size analysis, drug content, entrapment efficiency, in vitro release characteristics and also for stability analysis at different temperature and humidity conditions. Average particle size was found to be 21.40 μ m. Drug content of SLMs determined by HPLC analysis was found to be 98.6 \pm 0.31% while entrapment efficiency achieved was 98.02%. Drug release from the final formulation was found to be 90.3% in 90 min. SLMs formulated with glyceryl monostearate and sodium taurocholate can be used for oral delivery of hydrophobic drugs with in-vivo study still to be explored.

Keywords: Sirolimus; Immunosuppressant; Solid lipid microparticulates; Hot homogenization technique; Biocompatible lipid microparticles; Glyceryl monostearate

Introduction

Sirolimus (Rapamycin, Rapammune) is a macrolide lactone produced by *streptomyces hygroscopicus*. It is an immunosuppressive agent used for the prophylaxis of renal allograft rejection. The drug was first isolated from a soil sample from Rapa Nui, an island in the south pacific, hence the prefix "Rapa". It is extremely hydrophobic having molecular weight 914.2 g/mol [1,2,3]. Sirolimus is neither calcineurin inhibitor (as are cyclosporine and tacrolimus) nor an anti metabolite (as are mycophenolate mofetil and azathioprine). Sirolimus has a novel mechanism of immunosuppressant action involving the suppression of T-lymphocyte proliferation through inhibition of the target of rapamycin protein kinase complex (TORC) [4]. Mammalian TORC is critical for cell cycle progression and cell proliferation. Blockade of TORC inhibits cytokine-mediated proliferation in T cells, B cells and mesenchymal cells, including smooth muscle cells [5].

In recent years, biocompatible lipid micro- and nanoparticles have been reported as potential drug carrier systems as alternative materials to polymers [6]. They can be considered as physiologically compatible, physicochemically stable and allowing a large-scale production at a relative low production cost than liposomes [7]. These micrometer-sized particles consist of a solid fat core based on naturally occurring lipids and stabilized by a layer of surfactant molecules on the surface [8]. SLM are characterized by their better bio-compatibility as compared to competing polymeric microparticles [9]. Solid lipid micro- or nanoparticles are in the form of solid lipids and provide an alternative option for encapsulating lipophilic compounds. Protein stabilization could be achieved by a suitable particle formulation or a polymer mixture [10]. Different methods can be adopted to prepare solid lipid micro- or nanoparticles. In one of the study, modified high shear homogenization and ultrasound method was utilized to formulate SLNs [11]. Reithmeier H and co-workers [12,13] in two different studies prepared peptide containing glyceryl palmitate microparticles as well as lipid microparticles by using modified solvent evaporation method and a melt dispersion technique without the use of organic solvent.

In this study, we prepared solid lipid microparticles by hot homogenization method [14,15]. The main aim of this investigation was to develop and evaluate solid lipid microparticles of sirolimus for oral delivery. The proposed sirolimus concentration in this formulation was chosen after assay and moisture content correction of API (sirolimus). Furthermore, in the present investigation, we aimed at fabricating SLM of sirolimus by using hot homogenization method and with easily available solid lipids such as glyceryl monostearate (GMS) and characterized it for assay and in vitro release.

Materials and Methods

Materials

Sirolimus (Zydus Cadila, Ahmedabad); Glyceryl monostearate (Fine organics Ltd., Mumbai, India); Sodium taurocholate, Acetone, Ethanol (All AR grade) and Acetonitrile (ACN), H₃PO₄ (HPLC grade) were purchased from S.D. fine chemicals (Mumbai, India). All the excipients and reagents were used as received. Double distilled water was prepared freshly when ever required.

Preparation of solid lipid microparticles

Solid lipid microparticles were prepared by a hot homogenization technique. The formulations were optimized by first preparing blank microparticles by varying the content of GMS and sodium taurocholate and also by varying the processing parameters as shown in Table 1 and 2. Blank SLMs were prepared by first dissolving GMS in a mixture

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Ingredients	LIPO F/P 8001	LIPO F/P 8002	LIPO F/P 8003	LIPO F/P 8004	LIPO F/P 8005	LIPO F/P 8006
Glyceryl mono stearate	2.44 g	2.44 g	2.44 g	2.44 g	5 g	5 g
Sodium tauro cholate	0.1%w/v	0.2% w/v	0.3% w/v	0.1% w/v	0.2% w/v	0.2% w/v
Acetone	5ml	5ml	5ml	5ml	2ml	2ml
Ethanol	5ml	5ml	5ml	5ml	2ml	2ml
PVA solution	150 ml	150 ml	150 ml	-	-	-
Water	-	-	-	200 ml	250 ml	250 ml
Mannitol	-	-	-	-	-	2.5 g

Table 1: Formulation table for blank SLM formulations.

Formulation	Homogenization	Stirring time and speed	Centrifugation	Lyophilization
Lipo F/P 8001	-	30 min, 500rpm	-	-
Lipo F/P 8002	-	30 min, 500rpm	-	-
Lipo F/P 8003	-	60 min, 500 rpm	-	-
Lipo F/P 8004	12,000 rpm, 2-3 min	60 min, 500 rpm	-	-
Lipo F/P 8005	16,000rpm, 2-3 min	60min, 700 rpm	-	-25°C, 5.0 Pa for 17 hrs
Lipo F/P 8006	19,000 rpm, 2-3 min	90min, 1000rpm 90min, 1500rpm	5000 rpm, 10°C, 10 min	-25°C, 5.0 Pa For 17 hrs

Table 2: Processing variables for blank SLM formulations.

Bank SLM Formulations	Observation
Lipo F/P 8001	<ul style="list-style-type: none"> Very less amount of SLMs were observed. Crystals were formed. Physically Unstable formulation. After 2-3 days SLMs were disappeared.
Lipo F/P 8002	<ul style="list-style-type: none"> Very less amount of SLMs were observed. (but amount of SLMs were more in Lipo F/P 8002 than Lipo F/P 8001). Crystals were formed. Physically Unstable formulation. After 2-3 days SLMs were disappeared
Lipo F/P 8003	<ul style="list-style-type: none"> Very less amount of SLMs were observed. (amount of SLMs were very less in Lipo F/P 8003 than Lipo F/P 8001 and Lipo F/P 8002). Crystals were formed. Physically Unstable formulation. After 24 hrs SLMs were disappeared
Lipo F/P 8004	<ul style="list-style-type: none"> Amount of SLMs were more than Lipo F/P 8001, 2, 3 observed. Fewer amounts of crystals were formed. Physically Unstable formulation. After 10 days SLMs were disappeared
Lipo F/P 8005	<ul style="list-style-type: none"> Satisfactory amounts of SLMs were observed. Fewer amounts of crystals were formed. Physically stable formulation.
Lipo F/P 8006	<ul style="list-style-type: none"> Higher amounts of SLMs were observed. (more than Lipo F/P 8005) Crystals were not formed. Physically stable formulation.

Table 3: Observation of different trials of blank SLMs.

of acetone and ethanol (1:1). Organic solvents were completely removed using Buchi rotoevaporator (Buchi Labortechnik AG, Flawil, Switzerland). Lipid layer was melted by heating 5°C above the melting point of lipid. An aqueous phase was prepared by dissolving sodium taurocholate in double distilled water and heating to the same temperature as the oil phase. The hot aqueous phase was added to the oil phase, and homogenization was performed using Ultra Turrax® high speed homogenizer for 2-3 min. Obtained micro emulsion was immediately dispersed in cold water (2-3°C), under mechanical stirring. After a certain period, the samples were centrifuged at 10°C, 5000 rpm for 10 min. Obtained micro emulsion was then lyophilized using Virtis ADVANTAGE, Wizard 2.0 Lyophilizer and obtained lyophilized powder was further evaluated. From the results obtained, blank formulation Lipo F/P 8006 was selected for final drug loaded SLM formulation (Table 3 and 4). Drug loaded SLMs were prepared by dissolving the sirolimus and GMS in a mixture of acetone and ethanol and the same method was followed as for the blank preparation.

Particle size analysis

Particle size of the SLMs was determined immediately after dispersion, 1h later, and after 17h. The oily dispersible solution was resuspended in distilled water, prior to particle size determination. In a typical experiment, 150µl of the oily solution was mixed in 5 ml distilled water at 37°C, using vortex for 30s. Then the particle size of the obtained suspension was determined at 37°C for 200s by a Coulter N4 MD submicron particle size analyzer.

Assay (By HPLC)

Standard Preparation procedure: 25 mg of sirolimus pure sample was accurately weighed and dissolved in 100ml of acetonitrile. 4 ml of the above stock solution was then diluted in 25 ml of acetonitrile to obtain a concentration of 40µg/ml. 20µl of this standard solution was injected by Hamilton 100µl syringe. The response of the preparation in terms of area under the two major peaks corresponding to isomer-C, Sirolimus standard was measured.

Sample preparation procedure: 10 capsules containing lyophilized powder of sirolimus lipospheres equivalent to 10 mg of drug was taken in 250ml of volumetric flask and 150 ml of acetonitrile was added to it. It was then sonicated and volume was made up to 250ml with acetonitrile. The solution was filtered with 0.45µm filter paper and filled in the HPLC and measured for assay.

1. Mobile phase: 0.01% H₃PO₄; ACN (1:1)
2. Column: Kromasil/ C₁₈ (150mm x 4.6mm) 5µm.
3. Flow rate: 2.0ml/min
4. Detector: 277nm
5. Column temperature: 50°C
6. Sample cooler: 15°C
7. Injection volume: 20µl
8. Retention time: Sirolimus - 11.5min, Isomer C - 12.2 min

Entrapment efficiency

The drug entrapment efficiency was determined by suspending known quantity of SLMs in methanol and stirring for few minutes. The free drug (unentrapped) which got dissolved in methanol was separated by centrifugation using a refrigerated centrifuge that runs for 10 min at 5000 rpm/min. The obtained methanolic phase was filtered through a 0.8 µm membrane filter. Then the filtrate was analyzed by HPLC. The drug entrapment efficiency (EE) of the SLM was calculated by the equation:

$$EE(\%) = \left(\frac{W_{\text{initial drug}} - W_{\text{free drug}}}{W_{\text{initial drug}}} \right) \times 100$$

Where $W_{\text{initial drug}}$ = Weight of drug added to the system,

$W_{\text{free drug}}$ = Weight of free drug in the methanolic phase of system.

In vitro drug dissolution studies

Standard Preparation procedure: 25 mg of Sirolimus pure sample was accurately weighed dissolved in 100 ml of Acetonitrile. 2 ml of the above stock was then diluted in 250 ml of 0.4% SLS to obtain a concentration of 2µg/ml. 20µl of this standard solution was injected by Hamilton 100µl syringe. The response of the preparation in terms of area under the two major peaks corresponding to isomer-C, Sirolimus standard was measured.

Sample preparation procedure: In vitro dissolution was carried out by with USP type I apparatus using 500 ml of 0.4% SLS as the dissolution medium. 1 capsule (Microspheres equivalent to 1 mg of drug filled in each capsule) was placed in the each dissolution jar of the apparatus; dissolution was carried out at 120 rpm at 37°C for 90 mins. Known volume of the samples were withdrawn at predetermined time intervals and filled in HPLC vials, replaced with the same amount of fresh medium.

- 1 Mobile phase: 0.01% H₃PO₄; ACN (30:70)
- 2 Column: Kromasil/ C₁₈ (150mm x 4.6mm) 5µm.
- 3 Flow rate: 0.5ml/min
- 4 Detector: 277nm
- 5 Column temperature: 50°C
- 6 Sample cooler: 15°C
- 7 Injection volume: 20µl
- 8 Retention time of sirolimus: 1min

Scanning electron microscopy

Scanning electron microscopy (SEM) is an electron optical imaging technique that provides photographic images and elemental information. SEM is useful for characterizing the morphology and size of microscopic specimens with particle size as low as 10⁻¹⁰ to 10⁻¹² m. The sample is placed in an evacuated chamber and scanned in a controlled pattern by an electron beam. Interaction of the electron beam with the specimen produces a variety of physical phenomena that, when detected, are used to form images and provide elemental information about the specimens.

Scanning electron microscopy (JSM 840 A) was used to study the surface morphology of the solid lipid microspheres. The samples were analyzed after they had been gold sputtered using 25 nm gold film thickness. Both samples were studied at magnifications at probe current of 20 KV.

Short-term stability study

Sirolimus solid lipid lipospheres were stored at temperature 2-8°C,

25°C/60%RH and 40°C/75%RH for 30 days. Formulations at regular intervals were tested for physical changes, drug content and dissolution studies spectrophotometrically at 277 nm.

Results

Analytical method

Linearity of sirolimus in acetonitrile by HPLC: A simple method of estimation was developed in acetonitrile at 277nm. The linearity of sirolimus is shown in Figure 1.

Development of HPLC estimation method for sirolimus: A precise, sensitive, reproducible High Performance Liquid Chromatographic method of estimation for sirolimus was developed. Further estimation of sirolimus was carried out by using newly developed HPLC method. A mixture of acetonitrile and 0.01% phosphoric acid (1:1) was used as a mobile phase on a C₁₈ column. The flow rate was maintained at 2.0 ml/min. The peak height, area and retention time was calculated. The results are given in the Figure 2 and Table 5.

Particle size analysis

The effect of stirring speed on the properties of SLMs and the particle size distribution of SLMs is shown in Table 6 and 7 respectively.

Drug content using HPLC

Drug content of SLMs determined by HPLC analysis was found to be 98.6±0.31% (n=3)

Ingredients	LIPO F 8006
Sirolimus	101.10 mg
Glyceryl mono stearate	5 g
Sodium tauro cholate	0.2%w/v
Acetone	2ml
Ethanol	2ml
Water	250 ml
Mannitol	2.5 g

Table 4: Sirolimus loaded SLM formulation.

No	Name	Retention time(min)	Area (mcV*s)	Area %
1	Sirolimus	11.458	1698738	97.37
2	Isomer-C	12.142	45819	2.63
Total			1744557	100.00

Table 5: HPLC chromatogram parameters.

Formulation	Stirring speed (rpm)	Physical appearance	Particle size (µm)
Lipo F 8006	1000	Microparticulates, and free flowing	21.40
	1500	Agglomerated	40.20

Table 6: Effect of stirring speed on properties of SLMs of sirolimus.

Particle size range in µm	Midpoint size (d)	Frequency(n)	(n.d)	Average particle size in µm
0-10	5	0	0	21.40
10-20	15	56	840	
20-30	25	27	675	
30-40	35	15	525	
40-50	45	1	45	
50-60	55	1	55	
60-70	65	0	0	
70-80	75	0	0	
80-90	85	0	0	
90-100	95	0	0	
		Σn =100	Σ nd =2140	

Table 7: Particle size distribution of formulation LIPO F 8006 (stirring speed 1000 rpm).

Sr No.	Stability Conditions	Drug content (%)	% CDR
1	2-8°C	97.1%	87.5%
2	25°C/ 60%RH	92.2%	83.1%
3	40°C/ 75% RH	30.8%	25.3%

Table 8: Drug content and amount of drug release of SLMs at different stability conditions.

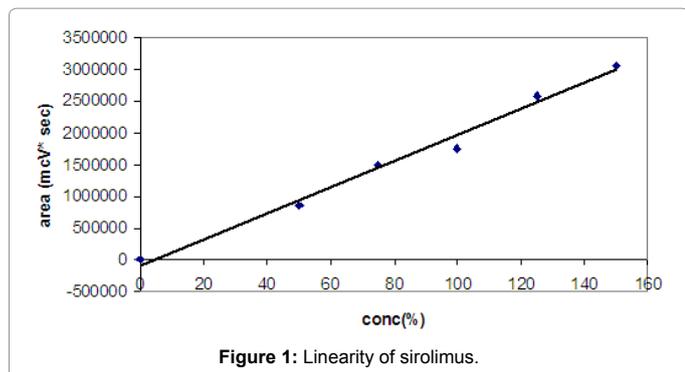


Figure 1: Linearity of sirolimus.

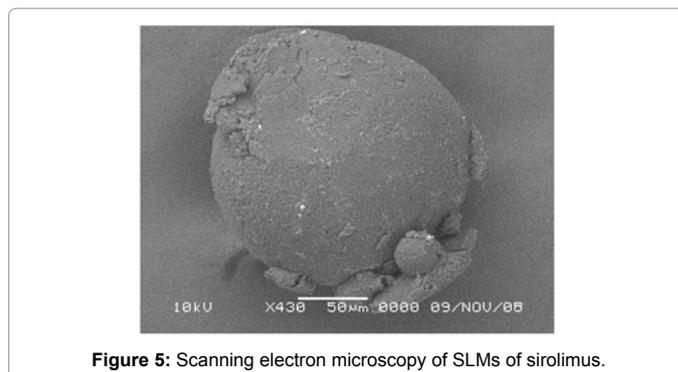


Figure 5: Scanning electron microscopy of SLMs of sirolimus.

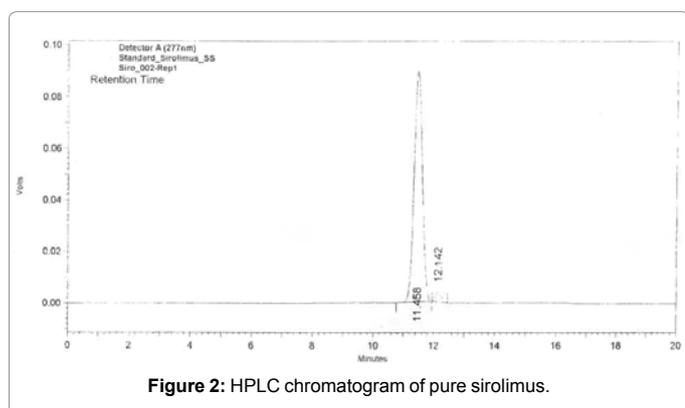


Figure 2: HPLC chromatogram of pure sirolimus.

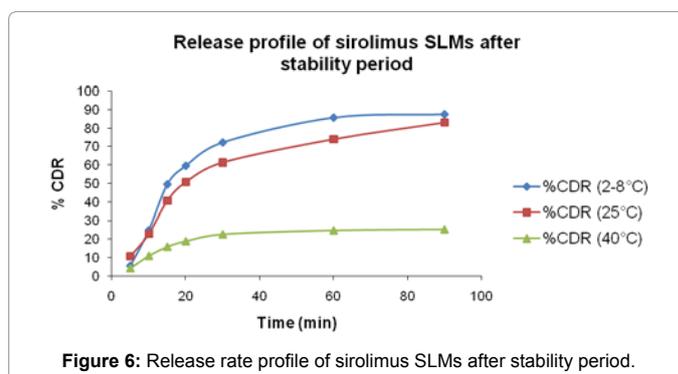


Figure 6: Release rate profile of sirolimus SLMs after stability period.

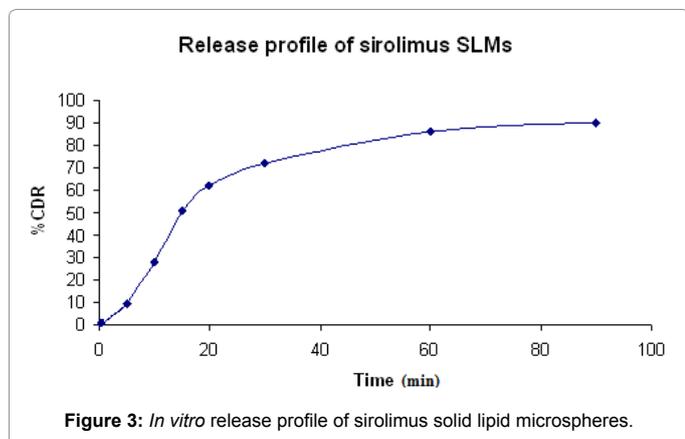


Figure 3: *In vitro* release profile of sirolimus solid lipid microspheres.

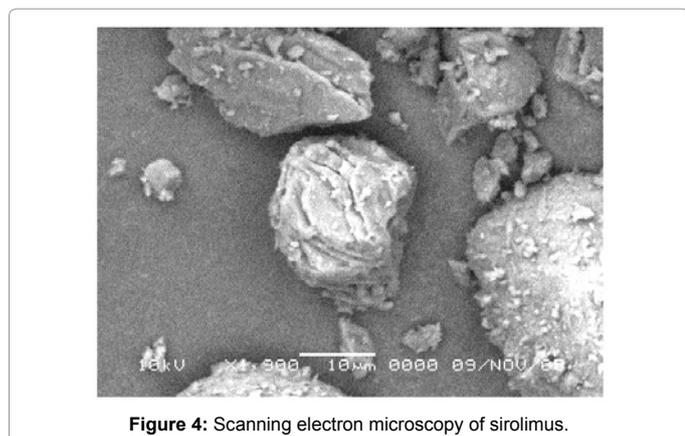


Figure 4: Scanning electron microscopy of sirolimus.

Entrapment efficiency

The entrapment efficiency (at zero day) of the microparticles (Lipo F 8006), was found to be 98.02%.

In vitro dissolution study

The dissolution study was carried out for the microspheres using 1 mg equivalent of drug in 0.4% SLS as dissolution media. The composition of dissolution media is (400mg of sodium lauryl sulphate in 1000 ml distilled water) and pH is 9. The release profile of solid lipid microparticles is shown in Figure 3. Amount of drug released within 90 mins was found to be $90.3 \pm 0.58\%$ (n=3).

Scanning electron microscopy

Scanning electron microscopy (SEM) studies were carried out to observe the surface morphology. Scanning electron microscopy (SEM) studies were carried out for pure drug as well as SLMs of sirolimus. SEM picture revealed that the pure drug was somewhat spherical with rough surface while the Sirolimus SLM was smooth and spherical. (Figure 4, 5)

Stability analysis of solid lipid sirolimus microsphere using HPLC

A precise, sensitive, reproducible HPLC method of estimation for sirolimus in the formulations was developed. The analysis was carried out soon after preparation and after 30 days (at 2-8°C, 25°C/60%RH and 40°C/75%RH). Drug content after the stability period found is reported in Table 8. Amount of drug release from the SLMs of sirolimus is also reported in the Table 8 and is graphically depicted in the Figure 6.

Discussion

In order to select the optimum formulation variables and to

optimize the processing parameters, blank SLMs were prepared. Particle size analysis of blank SLMs shows that the optimum concentration of emulsifier (sodium taurocholate) to get the uniform sized microparticles is 0.2%w/v. An increase or decrease in concentration leads to the crystal formation. Particle size may also get affected due to the homogenization speed and the stirring rate (Table 2,3). Particle size gets decreased as the stirring rate increases. The optimum stirring speed was found to be 1000rpm for 90 mins avoiding the crystal formation which is observed in case of the formulations with lower stirring rate and time.

SLMs were prepared by incorporating different amount (2.44g and 5g) of GMS. The higher amount of GMS showed satisfactory results. The higher drug content and entrapment efficiency with GMS is attributed to the high hydrophobicity resulting in increased accommodation of lipophilic drugs. Formulation Lipo F 8006 was subjected to *in vitro* release studies. The release study was performed in 0.4% SLS as dissolution media. The studies revealed that about 90.3 % of drug released from sirolimus SLMs after 90 mins at zero day. In vitro release study shows SLMs shows immediate release of the drug. After stability studies at different conditions (2-8°C, 25°C/60% RH, 40°C/ 75% RH) for thirty days, drug content and drug release profiles revealed that the formulations were most stable at 2-8°C and 25°C/60% RH but unstable at 40°C/ 75% RH (Table 8). Hence formulation should be kept at 2-8°C and 25°C/60% RH (cool and dry place). Presence of monoglyceride (GMS) may be responsible for the physical destabilization at higher temperature and humidity conditions. Lipids of less ordered crystal lattices such as GMS, though favored drug inclusion, were unstable due to the presence of monoglycerides.

Conclusion

Hot homogenization technique provides SLMs with higher drug content and entrapment efficiency by controlling the processing parameters. Hence, SLMs formulated with glyceryl monostearate and sodium taurocholate can be used for oral delivery of hydrophobic drugs with in-vivo study still to be explored.

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