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DESIGN AND DEVELOPMENT OF NEVIRAPINE LOADED SURFACTANT FREE CHITOSAN MICROEMULSION

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Abstract: Emulsification of liquid paraffin oil in aqueous solutions of chitosan without adding any additional surfactant is studied. The main objective of this study was to evaluate the dispersion of castor oil in aqueous phase in the presence of chitosan, and how this polymer promotes the stability of the obtained emulsions. Nevertheless, chitosan promotes emulsion production by increasing the matrix viscosity and provides stabilization of the oil-water interface by forming a dense hydrophilic polyelectrolytic brush on the water side of interface, which presents a significant barrier for coalescence – both steric and electrostatic. Chitosan stabilizes the emulsion mainly by the steric effect. These steric effects generate Van der Waals repulsion forces when two particles are too close. After loading with antiviral drug nevirapine, these emulsions were characterized in terms of phase contrast microscopy, hot stage microscopy, fluorescence microscopy, particle size, ξ potential, viscosity, entrapment efficiency and release studies using dialysis bag method. The prepared emulsions were stable in terms of mean globule size, change in drug content and retain they cationicity. The formulated emulsions are a promising carrier for nevirapine and other lipophilic drugs.

Keywords: chitosan, emulsion, nevirapine, surfactant

Emulsions are widely used in pharmacy, medicine, paints, food technology etc. Emulsification consists of dispersing one immiscible fluid into another *via* creation and stabilization of an interface. Therefore, such systems are thermodynamically unstable and may be kinetically stabilized by adding a further component or mixture of components that exhibit emulsifying properties (1).

The main advantage of this oral emulsion is that it is devoid of surfactant and most of the known efficient surfactants could be dangerous for health, especially upon parenteral administration, because of their hemolytic effects. They may alter cell membrane permeability leading to cell damage. Emulsifying agents such as surfactants reduce interfacial tension between oil and water. In the same way, many proteins are widely used in food industry to produce emulsion. Polysaccharides also may be used in preparation of emulsions but not all of them have high surface activity (1, 2). In fact, they promote the emulsion production by increasing viscosity of the water phase in which they are dissolved. In the same way, increasing the viscosity of the water phase (by addition of polysaccharide) slows down the diffusion of the dispersed droplets and therefore contributes to emulsion longevity. In this study, we were interested in nevirapine; (lipid soluble) used as a model drug and chitosan – a biopolymer (the second most abundant biopolymer after cellulose). It is a linear polysaccharide obtained by deacetylation of chitin. Chitin is not soluble in water due to the strong hydrogen bonding between aminoacetyl groups (3). By replacing the majority of these groups by the amine moieties, chitosan molecules can be dissolved in water, but only in acidic conditions. In such solutions, chitosan is a positively charged polyelectrolyte (cationic nature) due to protonation of the amine group (the NH_2 group in chitosan has a pKa of 6.4) (4–6). Chitosan has biological characteristics (hypolipidemic, hypocholesterolemic, mucoadhesion, water fat and dye binding capacity, nontoxicity, biodegradability) and physiochemical properties (polyelectrolytic, rheological thickening), which make it attractive for many

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potential applications in areas such as food and pharmaceutical industry and bioengeenering (7, 8). There are several papers available describing the stabilization of o/w emulsion where chitosan is adsorbed on the oil surface through interaction with an added surfactant (6–8). The aim of this study was to evaluate the dispersion of liquid paraffin oil in aqueous phase where chitosan was added without the help of any surfactant and how this polymer promotes the stability of the oral emulsion obtained. Chitosan allows the dispersion of oil by increasing the viscosity and provides stabilization of o/w interface, forming a dense polyelectrolytic brush on water side of the interface. Chitosan stabilizes the emulsion mainly by the steric effect. These steric effects generate Van der Waals repulsion forces when two particles are too close.

Oil-in-water emulsions are heterogeneous systems in which oil is dispersed as droplets in water; they represent an interesting prospect for the development of formulation for use as vehicle to deliver drugs orally to the human body. The potential pharmaceutical applications include a use as carrier for: poorly water-soluble drugs, sustained-release systems and site-specific drug delivery by binding ligands for various cell-surface receptors to the particle surface.

EXPERIMENTAL

Materials and methods

Chitosan with a broad molecular weight distribution of 10000 to 300000 g/mol was purchased from Sigma, Nevirapine was procured from Chromo Laboratories, India Private Ltd., Hyderabad. Chitosan was dissolved in 0.1 M acetic acid aqueous solution to a concentration of 2 wt%; under these conditions chitosan is largely dissolved by protonation of its amine groups (9–11). As a "model oil", liquid paraffin oil was provided by Ranbaxy Fine Chemicals Ltd. (RFCL). Its density and viscosity were determined to be 961 kg/m^3 and 0.985 Pa, respectively.

Preparation of surfactant free emulsion

Emulsions were prepared by homogenizing (12) liquid paraffin oil and chitosan solution during 4 min at room temperature using Ultra Turrex homogenizer at shear rate of 22000/s . The volume fraction of castor oil varied across the whole range between 0.5 to 1 mL. The concentration of chitosan varied between 0.5 to 2 wt%. The composition of optimized formulations is shown in Table 1.

Table 1. Composition of optimized formulations.

O/W ratio	Different % w/v of chitosan			
1.0/4.0 0.5/4.5 0.7/4.3 0.9/4.1	0.5	1	1.5	2

All formulations contain 1 mg of the drug

Phase contrast microscopy

It was carried out on Nikon (Eclipse E200, Japan), microscope with 40× magnification; few mL of sample was taken undiluted on glass slide and covered with cover slip. The precaution was taken to prevent drying of emulsion sample during observation.

ξ potential

The ξ potential was measured by a Zetasizer 2000 (Malvern Instruments). A sample was extemporaneously diluted in Milli-Q (Millipore Corp., USA) water (1 µL/10 mL) and injected in the apparatus. The measurements were carried out in the fully automatic mode. Each sample was analyzed twice.

Viscosity

The viscosity of the formulations were measured and analyzed using BohlinVisco 88 viscometer equipped with Bohlin software. Cone and plate geometry (cp5.4°/30) was employed with 0.05 mm gap. The samples were centrifuged free of bubbles, and an amount sufficient to fill the cone and plate gap completely was carefully scooped out and spread over the cone (≈ 1 mL). The ambient temperature was 25 ± 1°C.

Hot stage microscopy

To evaluate the stability of emulsion at different temperature, hot stage microscopy was performed .The microphotographs of emulsion were taken at 40 and 55°C through phase contrast microscopy at 40× magnification.

Table 2. Globule size and ξ potential of different concentrations of chitosan emulsions.

Chitosan conc. (%w/v)	ξ potential (mV)	Globule size (µm)
0.5%	+60.5 ± 7.99	4.50
1.0%	+71.8 ± 8.68	4.44
1.5%	+73.9 ± 8.07	2.10
2.0%	+74.6 ± 6.63	1.12

mV = millivolt; µm = micrometer

Fluroscence microscopy

The freshly prepared emulsions were observed under a fluroscence microscope (Leica) using FITC Dextran, a water soluble and fluorescent probe in water (purchased from Sigma).

Particle size analysis

The mean droplet size and particle size distribution of chitosan emulsion were determined by a laser diffraction particle size analyzer (Mastersizer®, Malvern, Orsay, France). The sample was extemporaneously dispersed in purified water at 2500 rpm until an obscuration rate of 5–18% was obtained. Background and sample were measured for 12 s. Each sample was measured in triplicate.

Transmission electron microscopy (TEM)

TEM was performed using negative staining with sodium phosphotungstate solution (0.2% w/v). Emulsions were dispersed in the staining solution for 30 min at the room temperature, placed on a copper grid covered with nitrocellulose, dried under vacuum for at least 24 h and observed under TEM.

Entrapment efficiency

Entrapment efficiency was calculated by determining the amount of un-entrapped drug (A2) after removal of un-entrapped drug by dialysis over the total amount of drug (A1). Entrapment efficiency was calculated using the equation:

$$E.E.\ (\%) = (A2 / A1) \times 100$$

***In vitro* drug release (dialysis method)**

The *in-vitro* release was determined using dialysis tubing (Sigma, USA). Free drug was removed from the formulation by exhaustive dialysis for 24 h against phosphate-buffered saline (PBS) buffer at 4°C. For the release experiment, 1 mL of emulsion was pipetted into a dialysis bag (Sigma, USA, MWCO: 12000), at room temperature. The dialysis bags were kept in 500 mL PBS at pH 7.4; temperature was maintained at 37°C at a shaking frequency of 150 rpm in dissolution apparatus. The samples were collected at different time durations viz. 0, 15, 30 min., 1, 2, 4, 6, 12 and 24 h. The release medium was exchanged with equal volume of fresh PBS solution. The concentration of released nevirapine was determined by performing high performance liquid chromatography.

Measurement of nevirapine

Nevirapine concentrations in the samples were measured by reversed phase HPLC. A Shimadzu HPLC apparatus with an attached UV/visible detector and a stainless steel C_{18} column (2.5 × 4.6 mm) were used. Mobile phase consisted of phosphate buffer and acetonitrile (ACN) in the ratio of 60 : 40, v/v. The pH was adjusted to 7.5 with 1 M NaOH. Both the components were filtered and degassed before use. The injection volume was 10 µL and detection was at 260 nm. The retention time of nevirapine was 5.5 min when the flow rate was kept at 1.0 mL/min and room temperature was maintained. Data were acquired and processed using class VP (Shimadzu) software.

Stability assessment

The storage stability of emulsion of paraffin in aqueous solution with concentration of chitosan varying between 0.5 and 2% w/v, with an equal 0.5 volume fraction of paraffin was observed. The droplet size distributions were monitored over periods of time stored at 4°C and 37°C. The creaming and the phase separation were assessed visually at given time intervals. All other visible changes were recorded. To evaluate its mechanical and physical resistance, the emulsion was subjected to an accelerated mechanical stress and its globule size distribution was measured before and after shaking at 100 strokes per min over 48 h at room temperature.

Phase separation and creaming:

Visual inspection was carried out at different time periods to assess the creaming in all formulations. This study was carried out up to 60 days in optimized formulations both at 4°C and 37°C.

Particle (globule size)

The globule size of optimized formulations was determined on storage for different time periods to assess the storage stability. This study was performed for about two months at room temperature. However, this study was also conducted by storing the formulations at 4°C.

pH of the formulation

An MP220 pH meter (Mettler Toledo, UK) was used for the determination of the pH value of the emulsions at room temperature (25°C ± 2).

RESULTS AND DISCUSSION

Creaming was dependent on the volume fraction of liquid paraffin, the lower was the volume fraction the faster was creaming, which is simply the effect of steric hindrance of droplets rising through the viscous medium. Droplet size distribution was

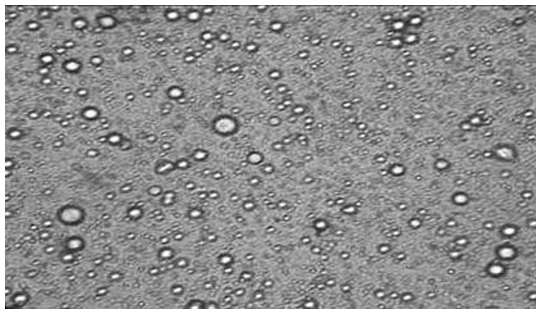

Figure 1. Phase contrast microscopy of 0.5%w/v chitosan emulsion at 40×

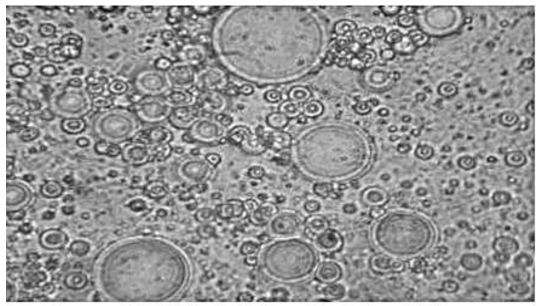

Figure 2. Hot stage microscopy of 0.5%w/v chitosan emulsion at 40×

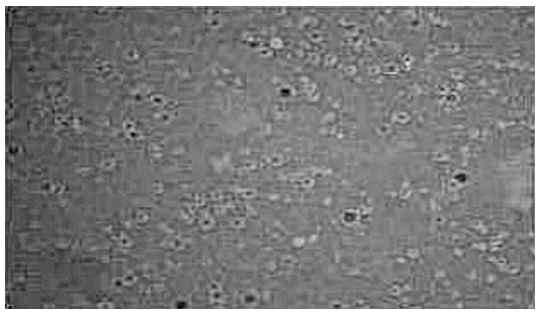

Figure 3. Fluorescence microscopy of chitosan emulsion at 40×

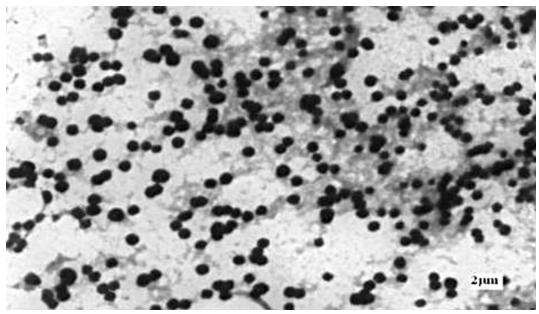

Figure 4. Transmission electron microscopy showing globule of 2 μm size

Table 3. Viscosity of different concentrations of chitosan emulsions at varying shear rates.

Shear rate	Viscosity (Pa)			
(1/s)	2%	1.5%	1%	0.5%
24.83	0.24	0.174	0.1	0.0086
42.1	0.32	0.185	0.12	0.0089
60.47	0.28	0.143	0.088	0.0075
78.93	0.25	0.132	0.082	0.0069
97.16	0.23	0.122	0.075	0.0065

Table 4. Entrapment efficiency of different formulations.

Formulation (% w/v)	Entrapment efficiency
0.5	68.49%
1.0	83.99%
1.5	85.88%

independent of the liquid paraffin volume fraction, essentially, the droplet size is determined by the function of continuous phase viscosity. For the high volume fraction of oil the emulsion was not stable, the droplets are of millimeter size (due to the dispersion of high viscosity chitosan solution in low viscosity majority phase) and their coalescence occurred immediately after the end of homogenization.

Through phase contrast microscopy photomicrographs and particle size analyzer graphs it is clearly seen that as the concentration of chitosan was increased it promoted fragmentation of oil into smaller droplets.

Phase contrast microscopy of different concentration of chitosan emulsions was done at 40× magnification. Morphologically, the suspended globules are oval to spherical in shape with somewhat hazy appearance of continuous phase in case of chitosan. The optimized formulations were found to be acceptable in terms of appearance and morphology as shown in Figure 1 (12).

Hot stage microscopy demonstrated that the emulsion formed were very stable. The emulsions were kept at varying temperature between 27°C and 55°C. At 40°C, emulsions were very stable and no change has taken place. At 55°C, the oil droplets

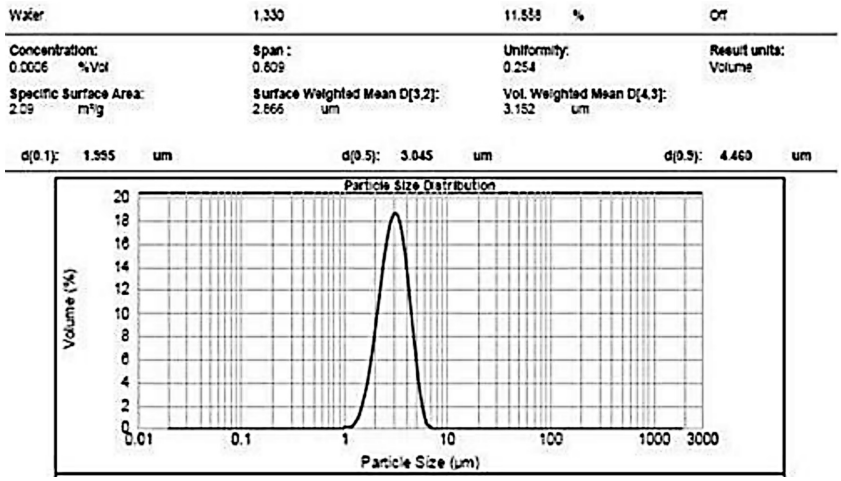

Figure 5. Globule size determination of 0.5% w/v of chitosan emulsion

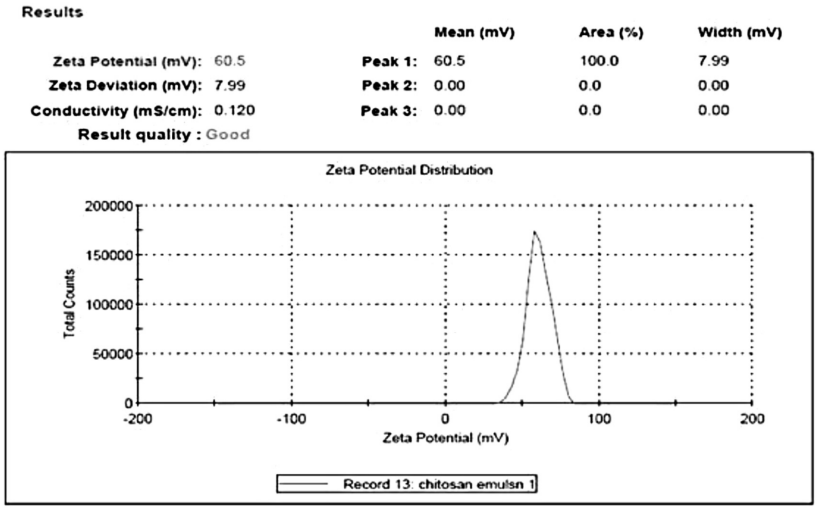

Figure 6. Measurement of ξ potential of 0.5% w/v chitosan emulsion

have become quite bigger and some of the droplets have ruptured and the aqueous phase has totally evaporated as shown in Figure 2.

Globule size of different concentrations of chitosan emulsion was determined by particle size analyzer and the size came out to be between 1 to 4 µm as summarized in Table 2. The largest droplet size was measured when the concentration of chitosan was the lowest. The higher was the concentration of chitosan, the smaller was the droplet size measured. Two % chitosan emulsion was of 1 µm and 0.5% of 4 µm size. In addition, microscopic observation suggests that the droplet size distribution was independent of the oil volume fraction (for the fixed chitosan content in water); essentially, the droplet size is determined by the local shear stress, i.e., the function of the continuous phase viscosity. We can clearly see that as chitosan concentration increases it promotes the fragmentation of oil into smaller droplets. And the oil droplets were of uniform size and were uniformly dispersed. Globule size of 0.5%chitosan emulsion is shown in Figure 5.

The formulations were subjected to determination of the ξ potential (Malvern zetasizer, UK) in

order to assess the contribution of cationic polymer on charge distribution of different formulations. It has been observed that all formulations showed positive ξ potential, i.e., all of them have positive charge. The value of ξ potential as depicted in Table 2 of 0.5 1, 1.5 and 2% chitosan formulations was found to be +60.5, +71.8, +73.9 and +74.6 mV, respectively. Positive ξ potential values are achieved in most of the emulsions prepared, this is helpful to develop high-energy barrier, which causes repulsion of adjacent droplets, resulting in the formation of stabilized emulsions. The ξ potential and standard deviation of the best formulation has been shown in Figure 6. The ξ potential is the difference in potential between the surface of the tightly bound layer of ions on the particle surface and the electro-neutral region of the solution. When the ξ potential is relatively high (25 mV or more, absolute value) the repulsive forces exceed the attractive London forces. The particles are dis-

Table 5. Drug release profile of 86% of different concentrations of chitosan emulsions.

Chitosan conc. (86%w/v)	% Release
0.5%	72.00%
1.0%	36.30%
1.5%	24.70%

Table 6. Drug release profile of 80% different concentrations of chitosan emulsions.

Chitosan conc. (80%w/v)	% Release
0.5%	90.70
1.0%	77.00
1.5%	50.60

Table 7. Particle size after different stability storage conditions.

		Particle (globule) size (µm)			
		0.5%	1%	1.5%	2%
Initial	d (0.1) d (0.5) d (0.9)	1.9 3.0 4.4	1.8 3.0 4.4	0.7 1.3 2.1	0.8 1.0 1.1
15 days	d (0.1) d (0.5) d (0.9)	2.0 3.1 4.5	1.6 4.2 4.4	0.6 1.3 2.2	0.84 1.1 1.0
30 days	d (0.1) d (0.5) d (0.9)	2.1 3.3 4.4	1.8 3.5 4.5	0.6 1.35 2.2	0.86 1.0 1.2
45 days	d (0.1) d (0.5) d (0.9)	2.1 3.2 4.45	1.88 3.4 3.9	0.7 1.36 2.3	0.88 1.2 1.3
60 days	d (0.1) d (0.5) d (0.9)	2.1 3.24 4.4	1.9 4.2 4.4	0.8 1.32 2.2	0.9 1.4 1.2

Abbreviations used: d = dimension in µm

Table 8. pH of different fotmulations stored at room temperature.

Batch	pH				
	Initial	15 days	30 days	45 days	60 days
0.5%	4.91	4.91	4.90	4.90	4.90
1.0%	5.00	5.00	5.10	5.10	5.11
1.5%	5.10	5.11	5.11	5.11	5.15
2.0%	7.23	7.23	7.23	7.33	7.35

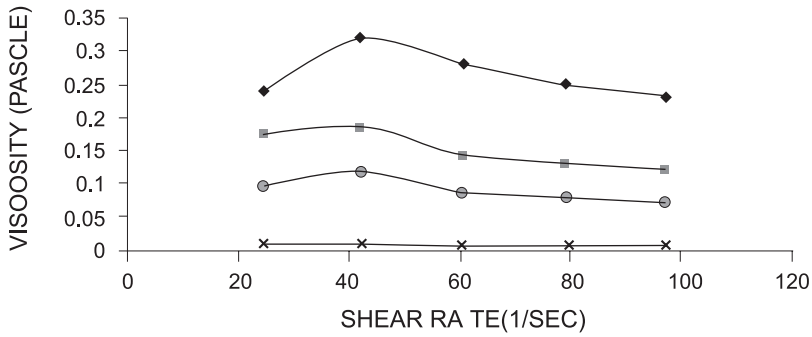

Figure 7: Viscosity measurement of different conc. of chitosan emulsion at varying shear rates; 2% chitosan emulsion (♦) ; 1.5% chitosan emulsion (■); 1% chitosan emulsion (●); 0.5% chitosan emulsion (×)

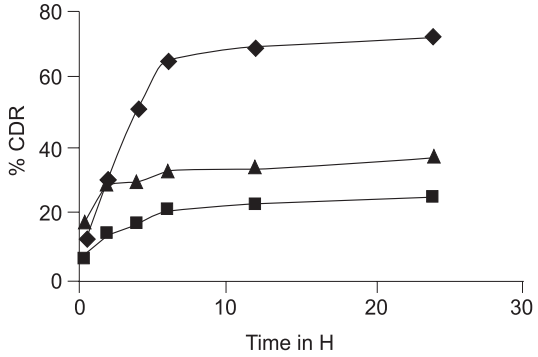

Figure 8. Drug release profile of nevirapine emulsion containing 86% w/v of chitosan for 24 h; 0.5% chitosan emulsion containing 86% of chitosan (♦); 1% chitosan emulsion containing 86% of chitosan (■); 1.5% chitosan emulsion containing 86% of chitosan (▲)

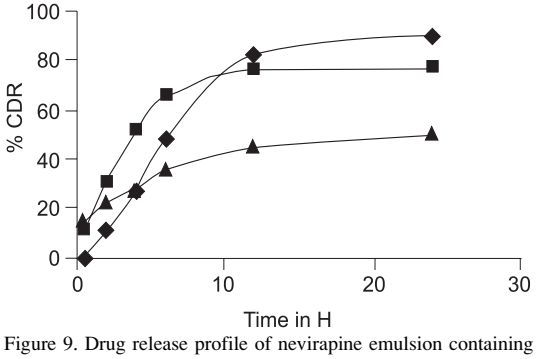

Figure 9. Drug release profile of nevirapine emulsion containing 80% w/v of chitosan for 24 h; 0.5% chitosan emulsion containing 80% of chitosan (♦); 1% chitosan emulsion containing 80% of chitosan (■); 1.5% chitosan emulsion containing 80% of chitosan (▲)

persed and the system is deflocculated. On the other hand, when the ξ potential is low (less than 25 mV, absolute value), the attractive forces exceed the repulsive forces, and the particles come together leading to flocculation.

Chitosan promotes the emulsion production by increasing the viscosity of the water phase. The viscosity of different concentrations of chitosan at varying shear rates is shown in Table 3. The graph of dependence between shear rate and viscosity in Figure 7 shows that, as the concentration of chitosan is increased, viscosity increases simultaneously, i.e., 2% has the highest viscosity and 0.5% exhibits the lowest. As the shear rate is increased, viscosity first increases then, on further increase of shear rate, viscosity decreases, which shows that the emulsion exhibits pseudoplastic (shear thinning) behavior.

The formulations were loaded with FITC dextran to evaluate whether the emulsion formed was o/w or w/o. FITC dextran dye is water soluble; therefore, the continuous phase appeared green in color and the dispersed phase in white as presented in Figure 3, therefore the emulsion formed is o/w.

The average globule size of all the formulations was well controlled and found to be 2 μm by transmission electron microscopy. This shows that the size of the globules were only in micron range as shown in Figure 4.

For drug entrapment studies, formulation was kept in dialysis tubing overnight at 40°C in PBS (pH 7.4). Free drug was estimated in the release media. Drug entrapment is an important parameter because of the low aqueous solubility of nevirapine. The entrapment efficiency results are been shown in Table 4. They are in line with the assumption that nevirapine would be carried in the oil phase and that it lacks amphiphilic properties to acquire oil-water interface. The conclusion is that a chitosan emulsion can be used for solubilization of nevirapine and perhaps as a sustained release preparation.

Dissolution studies were performed for 24 h in dissolution test apparatus; PBS was taken as dissolution medium. Among 86% and 80% of different concentration of chitosan used, 0.5% formulation shows the best release profile as depicted in Figures 8 and 9 (Tables 5 and 6). Consequently, it may be due to the partitioning of the drug between the oily droplets and the external aqueous medium. Nevirapine, being highly lipophilic drug, diffuses out slowly from oily phase to external aqueous phase. The presence of chitosan in formulation further sustains the release profile, as it is also known to act as emulsifier and present at the interface, forming the matrix around the oily core.

There was no change in mean droplet size before and after storage of samples at room temperature and also emulsion was able to resist the thermic shock of the autoclaving cycle. This was also confirmed by the lack of change in mean droplet size during the excessive shaking (100 strokes per min over 48 h) of the emulsion. Initial particle size and minute change in particle size after different stability storage condition was observed (Table 7). The creaming rate and the height of creamed layer were found to be strongly dependent on the concentration of chitosan, which affects the droplet size and viscosity of the continuous phase. The network of chitosan chains reduces the droplet diffusion in concentrated solutions and stabilizes the contact between droplets, improving the emulsion stability. It was seen that as the chitosan concentration increases, the stability of emulsion also increases simultaneously. Hence, 2% w/v chitosan emulsion was the most stable emulsion. The emulsion was also stable both physically and chemically following storage at 37^{o}C. There was no significant change in pH of different formulations when stored at room temperature. Initial pH and minute change in pH, if any, has been shown in Table 8 on storage for about two months.

The value of ξ potential and mean droplet size did not differ markedly from batch to batch, indicating that the experimental conditions were well controlled. The emulsions were found to be stable with respect to phase separation and creaming. It was also found that there was no phase separation and creaming up to 60 days in optimized formulations both at 4^{o}C and 37^{o}C.

Thus, it is possible to have stable emulsions of liquid paraffin oil (used here as a model oil) in the aqueous solution by adding only chitosan without any additional surfactant. Therefore, through this study it is proved that not only surfactant can produce a stabilized emulsion but if the viscosity parameter is taken into consideration, also a stable emulsion can be produced. Thus, different kinds of emulsions with different mechanical properties and shapes might be produced without the help of surfactant for the great benefit of environment.

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