MUCOADHESIVE SUBMICRON EMULSION: A NOVEL APPROACH FOR PERIODONTAL DISEASES

SHILPI PRASAD*[1], PREETI K SURESH[2]

[1] Astt.Professor, Siddhi Vinayaka Instt. of Technology and Sciences, Mangla, Bilaspur,C.G., India, [2]Reader, University Instt of Pharmacy, Pt. Ravi Shankar Shukla University, Raipur, C.G, India

ABSTRACT

The objective of present study is to formulate a local drug delivery of antimicrobial by sustained release delivery systems that can be used to treat periodontal disease. These systems are advantageous by maintaining optimum levels of antibiotic in the periodontal pocket for a sustained period of time, avoiding systemic side effects and ease of use with high patient acceptance.In the present work a mucoadhesive submicron emulsion (MuSME) of metronidazole has been developed and investigated for its *in vitro* drug release, *ex vivo* permeation effects and antimicrobial susceptibility against *Porphyromonas gingivalis*. The pseudo-ternary phase diagram was constructed using isopropyl myristate (IPM), Tween 80, lecithin and water. Chitosan is used as mucoadhesive agent. The permeation rates i.e. steady state flux of metronidazole from MuSME was found to be 1.2 times over that from a conventional formulation and 46.6 times over that from a 1% alcoholic solution of the drug.

Keywords: Mucoadhesive submicron emulsion, Isopropyl myristate, Metronidazole, Chitosan, Periodontal disease, Lecithin, Tween 80

INTRODUCTION

Periodontal diseases are inflammation and infection of the tissues and bones surrounding teeth.These disease occur due to bacteria from dental plaque which invades surrounding tissues resulting in the formation of pockets between gingiva and tooth.It causes gingival margin retraction and the development of an ideal environment for the growth of anaerobic bacteria responsible for the disease. The progression of this destructive process can cause tooth loss[1,2,3,4] The therapeutic goal is the removing of bacteria responsible for the inection by mechanical cleaning and topical application of antimicrobial agents, such as tetracycline, metronidazole,clindamycin, chlorhexidine,triclosan,amoxicillin and cetylpyridinium[5].Tetracyclines, in particular doxycycline, are used extensively in the treatment of periodontal disease, but the development of bacterial resistance has motivated for the use of metronidazole, very selective agent against anaerobic bacteria [6,7,8,9].Oral administration of antimicrobial agents has some disadvantages like hypersensitivity, gastrointestinal intolerance, development of bacterial tolerance[10,11]. Local delivery of antimicrobial agents is becoming more prevalent as it leads to higher concentration of the drug at the intented site of action using a lower dose with an associated reduction in side effects [12].

The first delivery devices were hollow polymeric fibres filled with tetracycline [13, 14]. They were not adhesive and the application required surgical procedure sometimes supported by the use of cyanoacrylate glue [15]. Surgical aplication was still required in another successful attempt with doxycycline and bioabsorbable materials [16].

To date submicron emulsions have been shown to be able to protect labile drug, control drug release, increase drug solubility, increase bioavailability and reduce patient variability [17]. Furthermore, it has proven possible to formulate preparations suitable for most routes of administration (oral, parenteral, pulmonary, ocular, topical and vaginal) [18].Mucoadhesive polymers incorporated in formuations can place the drug in intimate contact with the mucus layer at the site of action. They are well studied for oral cavity [19]. In the present approach a mucoadhesive submicron emulsion of metronidazole was formulated with penetration enhancing effect in the gums, sustained action at a very low dose along with ease of application and better patient compliance. Chitosan is used as a mucoadhesive agent which has itself antibacterial action against the gram –ve bacteria *P.gingivalis* [20].

Metronidazole is a nitroimidazole anti-infective drug (antibacterial (systemic); antiprotozoal). It exists as white to pale yellow, odorless crystalline powder, with a molecular wt. of 171.16. The melting point is 159-163°C. It exhibits no optical activity. It is sparingly soluble in water and in alcohol; slightly soluble in ether and in chloroform at 25 °C [21, 22].The partition coefficient values of metronidazole indicated its lipophilic nature.

MATERIALS AND METHODS

The drug metronidazole was obtained as a gift sample from Aarti drugs Ltd. Mumbai. Chitosan was obtained from Coastal Lab, Nellore, Andhra pradesh. Soybean lecithin (Epikuron® 200, phospha-tidylcholine content above 95%) was from Lucas Meyer (Hamburg, G). Tween 80 (polyoxyethylene sorbitan monooleate) and Iso propyl myristate, was bought from Merck (Mumbai, India). All the reagents and chemicals used were of reagent grade.

Methods

Pseudoternary phase diagram

Phase diagram was constructed for determining the area under which monophasic zone can be formed and the range of % (w/w) of surfactant, oil and water for formulation development can be decided. Phase diagram was prepared by water titration method [23]. Different amount of Tween 80 and soya lecithin in the ratio of 2:1 was dissolved in varying amounts of isopropyl myristate in stoppered tubes. It was stirred until clear solution was obtained. Titrations were then preceded by adding water at constant temperature with vigorous stirring for a sufficient length of time until the onset of turbidity.
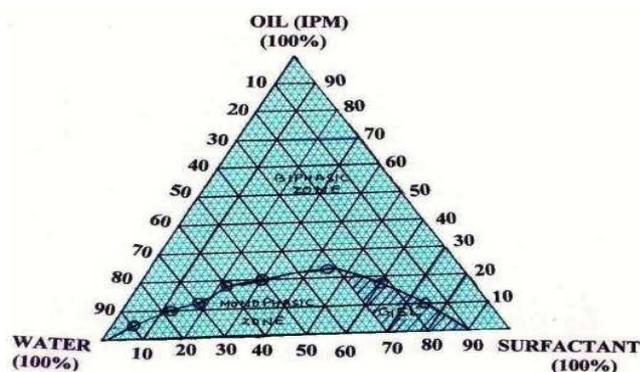

Fig. 1: Pseudoternary phase diagram of a system containing Isopropyl myristate (oil), lecithin, tween 80 and water

The pseudoternary phase diagram of the system containing isopropyl myristate (5%-23%), water (15%-90%), tween 80 and lecithin (5%-75%) is presented in the fig 1.

Preparation of O/W mucoadhesive submicron emulsion

Step1: Oil phase (10%): containing isopropyl myristate (5.26%) soya lecithin (3.33%), clove oil (0.01%), α- Tocopherol (0.4%) and drug (1%). Lecithin was mixed in the oil at 60℃ in a hot water bath with continuous stirring. When lecithin was dissolved, α-Tocopherol, clove oil and the drug was added with continuous stirring and heating.

Step 2: Aq. Phase (90%): containing Tween 80 (6.66%), Disodium EDTA (0.4%) and 0.5% Chitosan (or 1% or 1.5% chitosan in 1% acetic acid) was mixed over hot water bath with continuous stirring.

Step 3: The oil phase was mixed with the aqueous phase by magnetic stirrer for 10 min (or 15 or 20 min) at 300 rpm (or 400 or 500 rpm) at 60℃ (or 40℃ or 50℃) to prepare the coarse dispersion. It was furthur homogenized by ultrasonication for 15 min for removing any air bubbles and also for getting submicron emulsion. The temperature was maintained below 20℃.

The pH was adjusted to 6.75 with 0.1N NaOH.

Table 1: The formulation code and effect of variables on average globule size and percent drug entrapment of mucoadhesive submicron emulsions

S.No.	Formulation Code	Temperature (℃)	Time (min)	Stirring rate (rpm)	Chitosan (%w/w)	Sur +cosur (%w/w)	Average Particle size (nm)	Percent drug entrapment (%)
Variable 1 (Time)	CsSMEt1	50	5	300	1.5	5	856 ± 62.41	79.9 ± 5.55
	CsSMEt2	50	10	300	1.5	5	835 ± 56.7	81.1± 4.7
	CsSMEt3	50	15	300	1.5	5	825 ± 52.61	72 ± 2.34
Variable2 (surfactant %)	CsSMES1	60	15	300	1	5	257 ± 24.6	82.2 ± 2.37
	CsSMES2	60	15	300	1	10	365 ± 41.2	88.7 ± 3.67
	CsSMES3	60	15	300	1	15	395 ± 32.56	85.1 ± 5.45
Variable 3 (stirring rate)	CsSMER1	60	15	200	0.5	5	400 ± 42.48	85 ± 4.45
	CsSMER2	60	15	300	0.5	5	395 ± 40.3	86.7 ±2.35
	CsSMER3	60	15	400	0.5	5	390 ± 42.61	80.2 ± 1.15
Variable 4 (polymer %)	CsSMEP1	40	15	200	0.5	5	590 ±52.46	79 ± 6.3
	CsSMEP2	40	15	200	1	5	610 ± 42.48	81.4± 3.11
	CsSMEP3	40	15	200	1.5	5	638 ± 51.9	84.6 ± 5.34
Variable 5 (temperature)	CsSMET1	40	5	300	0.5	5	295 ± 23.13	75 ±1.22
	CsSMET2	50	5	300	0.5	5	290 ± 25.15	77.6 ± 1.25
	CsSMET3	60	5	300	0.5	5	257 ± 24.6	80.8 ± 1.56

Optimization of process variables

Various process variables (Temperature, Time of stirring, %age of surfactant,%age of polymer and rate of stirring) which could effect the preparation and properties of the submicron emulsions, were identified and studied. The method of preparation was accordingly optimized and validated. 15 formulations were designed on the basis of these variables. The effects of these variables were observed on average particle size and % drug entrapment of mucoadhesive submicron emulsions as given in table 1.

Particle size and size distribution

Particle size and size distribution of emulsions were determined by using Malvern Zetasizer NanoZS (Malvern instrument Ltd, UK), which works on non-invasive back scatters (NIBS) technology. All readings were taken in triplicate and the results indicate mean ± std.deviation.

Containment efficiency

The amount of drug present in the submicron emulsions was analyzed in terms of percent entrapment of drug.

Percent Entrapment of Drug (E):

2ml of mucoadhesive submicron emulsions were taken and 2ml of methanol was added to each formulation. They were ultra

centrifuged (Remi, India) at 20,000rpm for 30 min at 4℃. The supernatant was taken and after suitable dilutions with distilled water assayed for Metronidazole spectrophotometrically, at 310 nm. The percentage drug entrapment is calculated from the equation given below:

$$\% E = C_{Final} / C_{Initial}$$

Where, C_{Final} is the concentration of Metronidazole in the emulsion and $C_{Initial}$ is the original concentration of Metronidazole added during preparation.

The percent entrapments of Metronidazole in mucoadhesive submicron emulsions with different variables are presented in Table 1. All readings were taken in triplicate and the results indicate mean ± std.deviation.

Optimized Formulations

On the basis of effects of different process variables on particle size and % drug entrapment of mucoadhesive submicron emulsions the optimized formulations were selected as presented in Table 2 and presented in Fig 2 and 3 and they were further characterized for zeta potential, viscosity, mucoadhesive property and drug release profile.

Table 2: Optimized formulations and their selected parameters

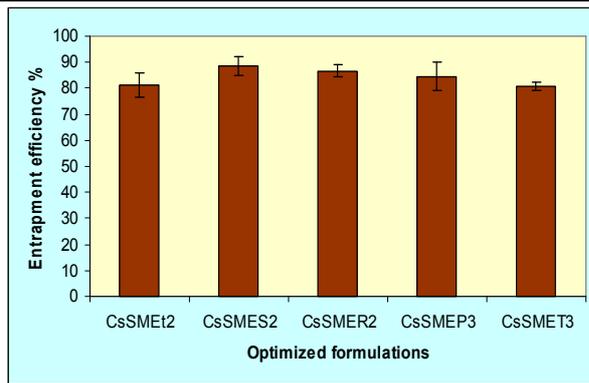| Formulation code	Parameter					Average particle size (nm)	% Entrapment
	Temp (℃)	Time (min)	Stirring rate (rpm)	Polymer concentration (%w/w)	Surf+ cosurf. (%w/w)		
1.CsSMEt2	50	10	300	1.5	5	835± 56.7	81.1± 4.7%
2.CsSMES2	60	15	300	1	10	365± 41.2	88.7±3.67%
3.CsSMER2	60	15	300	0.5	5	395± 40.3	86.7±2.35%
4.CsSMEP3	40	15	200	1.5	5	638± 51.9	84.6±5.34%
5.CsSMET3	60	5	300	0.5	5	257± 24.6	80.8 ± 1.56%

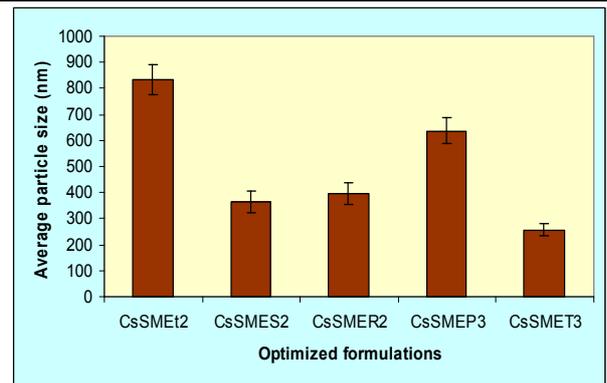**Fig. 2: Entrapment efficiency (%) of optimized formulations**

Fig 3: Average particle size (nm) of optimized formulations

Zeta Potential determination

The zeta potential is representative of particle charge. Zeta potential of optimized formulation was measured by electrophoresis, which was performed with a Malvern Zetasizer nanoZS (Malvern Instruments Ltd., UK). The Zeta potential cell was filled with a measured amount of sample and inserted with its integral gold electrodes close to the lid for analysis. The result is reported in table 3.

Table 3: Zeta potential, Viscosity, polydispersibility and mucoadhesive % of the optimized formulations

Optimized Formulations	Zeta potential(mV)	Viscosity (mPa-s)	polydispersity index (PDI)	Mucoadhesive %
1.CsSMEt2	-4.96 ± 0.88	39 ± 3.21	0.746	68±4.54
2.CsSMES2	-9.54 ± 0.67	30± 2.45	0.671	65±3.22
3.CsSMER2	-16.1±0.29	32±2.34	0.678	54±5.21
4.CsSMEP3	-16.0 ± 0.78	37±5.43	1.000	57±2.70
5.CsSMET3	-8.45 ± 0.89	28±2.76	0.737	66±3.25

[a]Note : all readings were taken in triplicate and the results indicate mean ± std.deviation.

Viscosity determination

The apparent shear viscosity of the emulsions was determined by Hoppler's rolling sphere viscometer at 20ºC.

Ex vivo Mucoadhesive determination

The adherent submicron emulsion droplets were quantified by analyzing the amount of drug in the non- adherent submicron emulsion.

Procedure

Porcine buccal mucosa with an area of 2.5 inch^2 was used for this purpose. It was obtained from a local slaughter house. It was defatted and preserved in normal saline solution till use. 1 ml of each of the formulations were applied on the inner side of the mucosa and the outer side was glued to the base of a beaker with cyanoacrylate glue and the beaker was filled with 100ml of simulated saliva [24] (pH 6.75) at 37ºC. It was left for 30min.By measuring the amount of drug (before and 30 min after application of formulations) with a UV spectrophotometer at 318.0nm, the mucoadhesive % was estimated by the following equation,

$Na = ((No-Ns)/No \times 100$

Where,   No = amount of drug applied,

               Ns = amount of drug released in simulated saliva after 30min.

The zeta potential, viscosity, polydispersity index and mucoadhesive % of the optimized formulations has been reported in Table 3and fig 4-6.
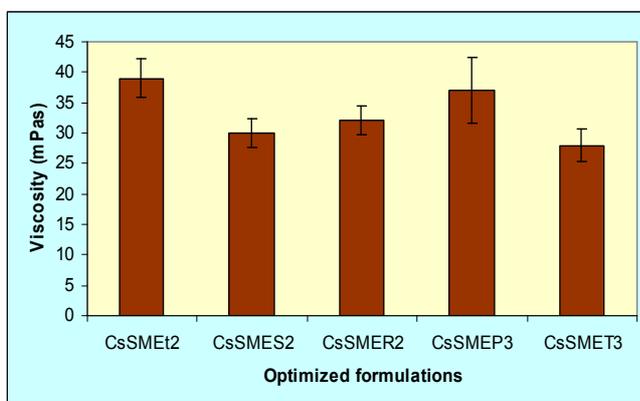

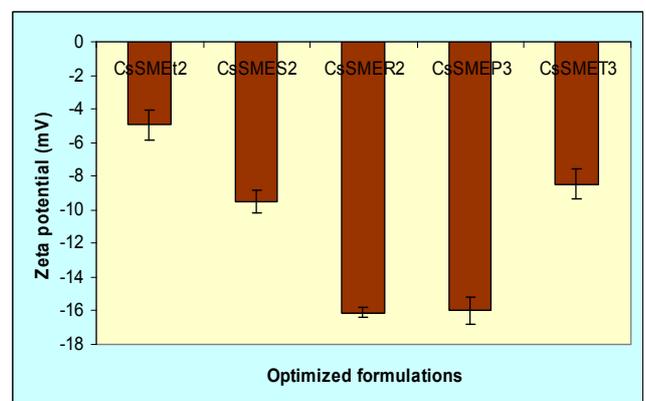**Fig 4: Viscosity (mPas) of optimized submicron emulsions**

Fig 5: Zeta potential (mV) of optimized submicron emulsions
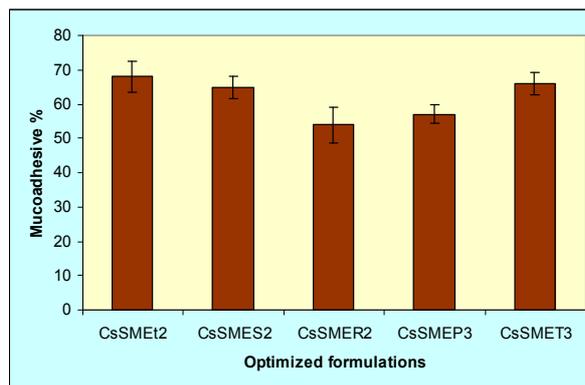
Fig. 6: Mucoadhesive (%) of optimized submicron emulsions

In vitro drug release profile

The in vitro drug release studies of metronidazole from various optimized formulations were performed by dialyzing (Dialysis membrane-60, 10000 MWCO. Hi-Media Ltd.) 2ml sample of different formulations against 100ml of simulated saliva (pH 6.75) at 37°C under moderate stirring conditions. At stipulated intervals of time 0.5ml of the sample was taken out and replaced with the same amount of simulated saliva maintaining sink conditions. The dialysate was measured for the presence of metronidazole spectrophotometrically at 318.0 nm over a period of 8 hrs. Final sample was taken after 24 hrs. Total % drug released after 8 and 24 hrs is reported in table 4 and presented in Fig 7.

Table 4: % drug released after 8 and 24 hrs of the optimized formulations

S.No	Optimized formulations	% drug released after 8 hrs	% drug released after 24 hrs
1	CsSMEt2	71.2±2.26	89.5±1.76
2	CsSMES2	56.29±1.96	93.11±2.11
3	CsSMER2	75.1±2.36	83.41±4.84
4	CsSMEP3	68.3±2.84	80.3±3.67
5	CsSMET3	71.2±1.56	85.98±3.45

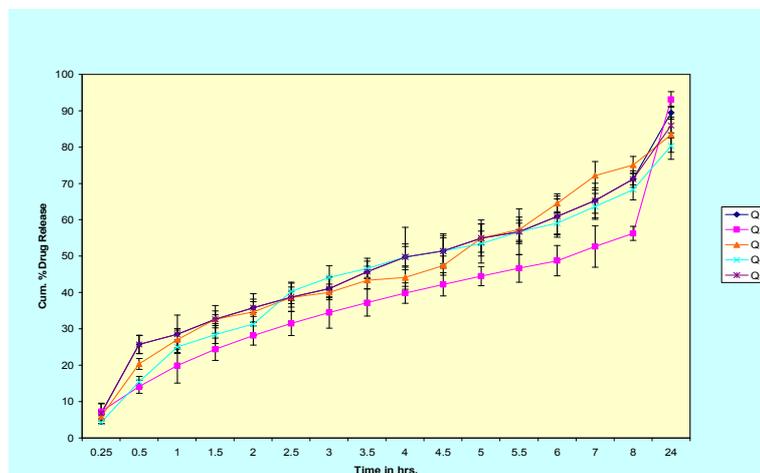[a]Note: All the values are the mean values ± S.D, n=3

Fig. 7: In vitro drug release of optimized formulations

Statistical data analysis

All the in vitro drug release studies were carried in triplicate and data were expressed as the mean value ± S.D. Data were analyzed by one-way analysis of variance (ANOVA). A multiple comparison test was used to compare different release parameters after 24 hrs, and a P value of 0.05 was considered to be significant.

Final Optimized Formulation

On the basis of the values of mucoadhesive %, viscosity, zeta potential, and in vitro release data, the final optimized formulation was selected as CsSMES2 and presented in table 5.

Table 5: Mucoadhesive %, viscosity, zeta potential, and in vitro release data of CsSMES2

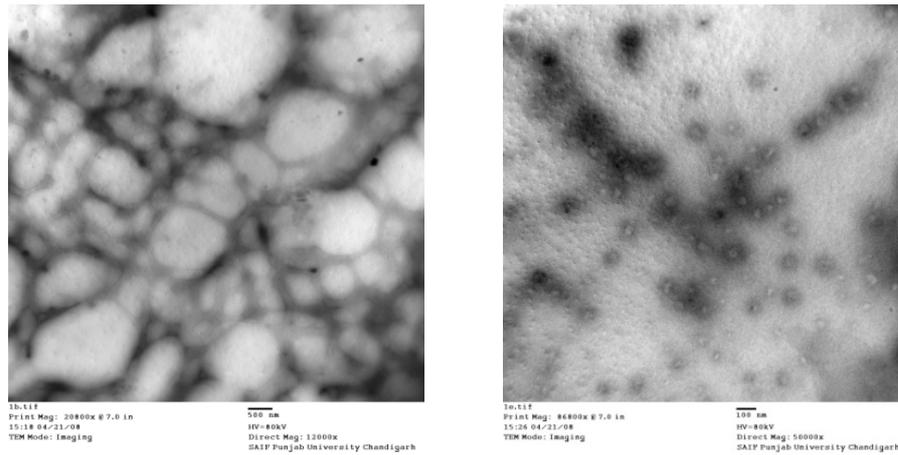| Formulation code	Zeta potential (mV)	Viscosity (mPa-s)	Poly-dispersity index	Mucoadhesive%	% drug released after 24 hrs
CsSMES2	-9.54±0.67	30± 2.45	0.671	65±3.22	93.11±2.11

Fig. 8: TEM Images of CsSME2 at 12000X and 50000X

TEM Images of CsSMES2

The TEM images of the final optimized formulation (CsSMES2) were shown at 12000X and 50000X magnification. It shows O/W submicron emulsions with polymer coating at the interface and is presented in Fig 8.

Ex vivo permeation studies

Ex vivo permeation studies (Li *et al*; 2005) were conducted on porcine buccal mucosa taking the optimized formulation, a conventional dental formulation and an alcoholic solution of metronidazole with the same concentration as the formulation i.e. 1% drug concentration.

Tissue preparation

The porcine buccal mucosa is largely used for in vitro experiments because the permeability of this membrane is very similar to the human buccal tissue. Porcine buccal mucosa with a fair amount of underlying connective tissue was surgically removed from the oral cavity of a freshly killed male pig, from a local slaughter house. The buccal mucosa was placed in ice-cold phosphate buffer (pH 7.4). The connective tissue of the mucosa was carefully removed using fine-point forceps and surgical scissors.

Ex vivo diffusion study

The ex vivo diffusion studies were carried out in modified Franz diffusion cells having $1.5cm^2$ diffusion area. The receptor compartment was filled with a volume of 50 ml of phosphate buffer saline pH (7.4) and was maintained at 37± 0.5ºC by means of a water bath. The solution in the receptor compartments was continuously stirred at 100 rpm using a Teflon coated magnetic stirrer. The porcine buccal mucosa, which was approximately 1-mm-thick, was clamped between the donor and receiving compartments. Two milliliter of the optimized formulation was placed in the donor compartment. The amount of metronidazole diffused through porcine buccal mucosa was determined by removing aliquots of 1 ml from the receptor compartments using a syringe and immediately replacing the same volume of phosphate buffer saline (pH 7.4, kept at 37± 0.5ºC). The samples were transferred to volumetric flasks, and stored in a refrigerator until they were analyzed. Sampling schedule was 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8 and 24 hrs. The same procedure was repeated with a dental conventional formulation and an alcoholic solution of metronidazole with the same concentration as the formulation i.e.1% drug concentration. All experiments were carried out in triplicate.

The cumulative amount of drug permeated per unit diffusion area through 24hrs has been presented graphically in Fig. 9.
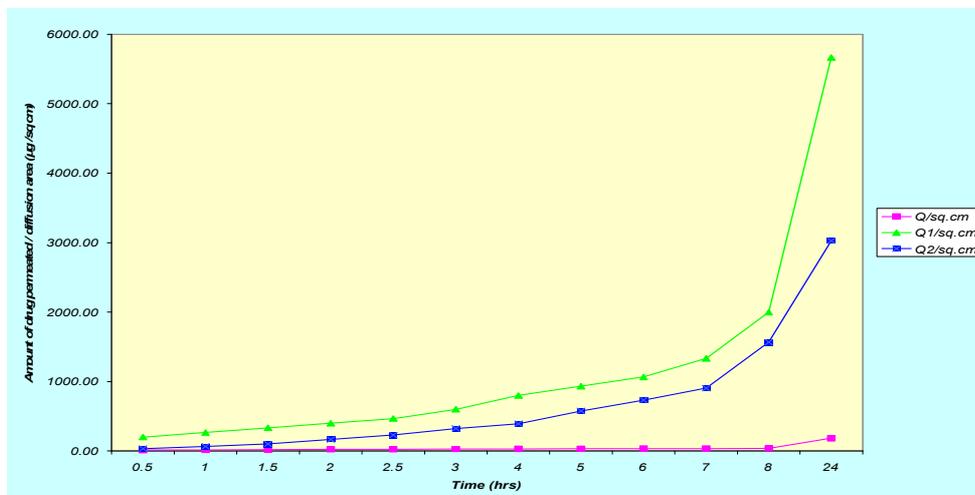

Fig. 9: Amount of drug permeated per unit diffusion area with respect to time (Q-1% alcoholic solution, Q1-CsSMES2, Q2-conventional dental formulation)

Table 6: The steady state flux (Js), permeability coefficient (Kp), Lag time (Tl), effective diffusion area and donor concentration (Cd) of different formulations

S.No	Formulations	Steady state flux J ($\mu g/cm^2/hr$)	Permeability coefficient Kp (cm/hr)	Lag time (T_l)(hr)	Effective diffusion area (cm^2)	Donor concentration C_d ($\mu g/cm^3$)
1	CsSMES2	133.3	0.0172	2	1.5	8870
2	conventional dental formulation	114.67	0.0115	3	1.5	10000
3	1% alcoholic solution	2.86	0.0007	8	1.5	10000

Data analysis

The absorption is a passive diffusion process and can be described by

Fick's law equation:

J = dQ /Adt

where J is the steady-state buccal mucosa flux in $\mu g/cm^2/h$, dQ is the change in quantity of material passing through the membrane into the receptor compartment expressed in μg, A is the active diffusion area in cm^2 and dt is the change in time. The steady-state flux J ($\mu g/cm^2/hr$) of metronidazole through the porcine buccal mucosa was calculated from the slope of the linear portion of the cumulative amount permeated through the membrane per unit area vs. time plot.

To determine the permeability coefficient, the following equation was used:

Kp = Js / Cd

where Kp is the permeability coefficient, Js is the flux calculated at the steady-time and Cd is the donor concentration.

The steady state flux (Js) and permeability coefficient (Kp) of the optimized formulation, a dental conventional formulation and an alcoholic solution of metronidazole with the same concentration as the formulation i.e.1% drug concentration is presented in table 6. Statistical analysis of the data was done at 95% level of significance

Statistical data analysis

All skin permeation experiments were done in triplicate and data were expressed as the mean value ± S.D. Statistical data were analyzed by one-way analysis of variance (ANOVA). A multiple comparison test was used to compare different formulations, and a P value of < 0.05 was considered to be significant.

Antimicrobial susceptibility test

P. gingivalis was isolated from subgingival plaque of adult periodontitis patients (Dental Deptt of CIMS, Bilaspur, C.G.,India). Plaque sample was cultured on Brain heart infusion agar supplemented with hemin (5 $\mu g/ml$) and vitamin K (0.1$\mu g/ml$). Identification of the microorganism was based on colony pigmentation, gram stain and biochemical tests [25]. Briefly, serial twofold dilutions of the microbial agents were prepared in Brain heart infusion agar broth containing hemin (5$\mu g/ml$) and vitamin K (0.1$\mu g/ml$). Three or four colonies of the organism were picked from an overnight culture on blood agar plate and inoculated into a tube of 5–6 ml supplemented with Brucella medium. This medium was enriched with hemin (5$\mu g/ml$) and vitamin K (0.1$\mu g/ml$) prior to sterilization and sodium bicarbonate (1 mg/ml) added just prior to use. After over night incubation at 37 °C, the culture was diluted in Brain heart infusion agar broth to the turbidity of 0.5 McFarland standard (10^8CFU/ml), and was then further diluted to 1:200. Samples containing the drug in a concentration range of 200-2000 $\mu g/ml$ were prepared and discs were charged with a capacity of 0.01ml. Discs were also charged with the sample containing the selected formulation diluted to 1:5 and 1:10. The plates were inoculated with the charged discs and were incubated at 37 °C in GasPak jars for approximately 48 h. An inoculated broth containing no antimicrobial agent was included as a growth control. Inhibition zone diameters were measured after 48 h incubation. The results are presented in table 7 and shown graphically in Fig 10. The photograph of the different samples is shown in Fig 11. All readings were taken in triplicate and the results indicate mean ± std.deviation.
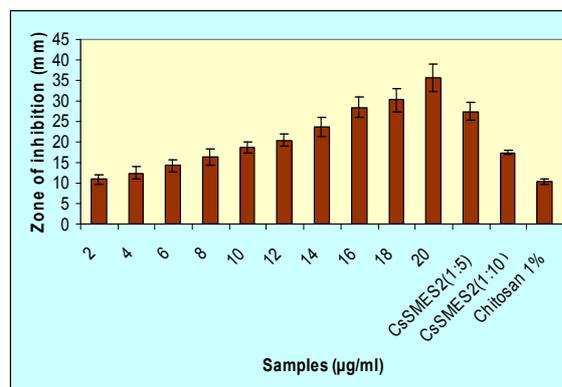
Fig. 10: Zone of inhibition of different dilutions of Metronidazole, formulation and chitosan

Table 7: The zone of inhibition of different dilutions of metronidazole, formulation and chitosan

S.No	Samples ($\mu g/ml$)	Zone of inhibition (mm)
1	0	0.00
2	2	10.9±1.23
3	4	12.5±1.5
4	6	14.2±1.4
5	8	16.4±2.1
6	10	18.7±1.3
7	12	20.5±1.4
8	14	23.7±2.3
9	16	28.5±2.6
10	18	30.2±2.8
11	20	35.6±3.4
12	CsSMES2(1:5)	27.5±2.3
13	CsSMES2(1:10)	17.4±0.5
14	Chitosan 1%	10.6±0.67

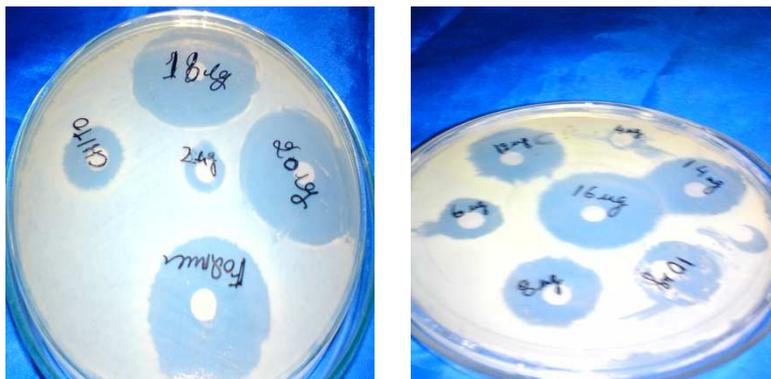[a]Note: The values indicate mean ± std dev, n=3

Fig. 11: Zone of inhibition of different samples

Stability of mucoadhesive submicron emulsion

Submicron emulsions are known to be thermodynamically stable systems. Once a submicron emulsion is formed, it can theoretically be stable forever. However, emulsions are not stable systems and they eventually will break down. The separation of the oil and water phases of an emulsion is a kinetic process. Usually when we say an emulsion is stable, it actually means the emulsion takes a long time to separate.

Effect of storage temperature on stability of emulsions

The optimized formulation CsSMES2 was stored in amber colored glass bottles at 4 ± 1°C, 25 ± 1°C and 45 ± 1°C for a period of 45 days and observed for effect of temperature on phase separation, average particle size, viscosity, zeta potential and % entrapment. The observations are recorded in Table 8 and the photomicrograph can be observed in fig 12(a) and (b).

Table 8: The effect of storage temperature on different parameters (sampleCsSMES2)

| Parameters	Temperature (°C)	Days Initial	15	30	45
Phase	4 ± 1	-	-	-	-
separation	25 ± 1	-	-	-	-
after days	45 ± 1	-	-	-	+
Average	4 ± 1	365± 41.2	365± 50.3	365± 48.9	365± 47.6
particle size	25 ± 1	365± 41.2	365± 40.1	365± 31.2	365± 47.3
(nm)	45 ± 1	365± 41.2	378± 47.2	385± 48.4	390± 46.3
Viscosity	4 ± 1	30± 2.45	30± 5.3	29± 4.9	29± 4.6
(mPa-s)	25 ± 1	30± 2.45	30± 4.1	29± 3.2	29± 4.3
	45 ± 1	30± 2.45	29± 4.7	29± 4.8	28± 4.6
Zeta	4 ± 1	-9.54 ± 0.67	-9.53± 0.34	-9.52± 0.63	-9.50± 0.34
potential	25 ± 1	-9.54 ± 0.67	-9.54± 0.76	-9.53± 0.71	-9.52 ± 0.61
(mV)	45 ± 1	-9.54 ± 0.67	-9.5± 0.51	-9.0 ± 0.93	-8.45±0.42
%	4 ± 1	88.7±3.67	88.5±3.4	88.4±1.2	88.0±3.5
Entrapment	25 ± 1	88.7±3.67	88.2±3.8	88.1±3.5	88.0±2.3
	45 ± 1	88.7±3.67	88.1±3.6	88.0±2.4	87.7±3.6

[a]Note: - No separation   + Slight Separation
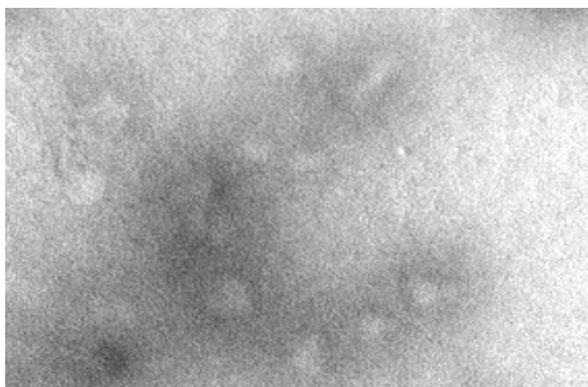
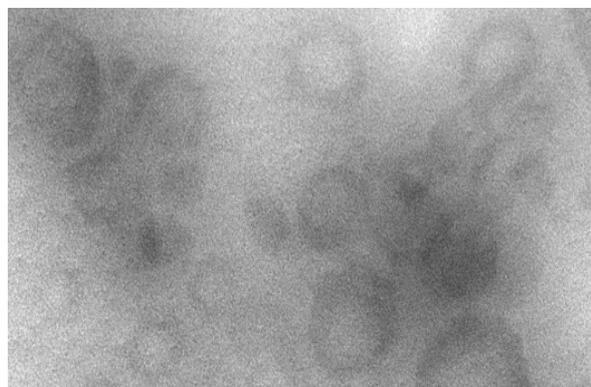**Fig 12(a): Photomicrograph of fresh formulation (CsSMES2)**

Fig12(b): Photomicrograph of CsSMES2 after storage for 45 days at 45 ± 1°C

Effect of centrifugation on stability of emulsion

Optimized formulation was subjected to centrifugation for 30 min, at room temperature. Samples was withdrawn after particular effect of centrifugation interval and observed for phase separation, average particle size, viscosity and % entrapment. The observations are recorded in Table 9.

Effect of agitation on stability of emulsion

The effect of agitation on the stability of emulsion was examined by agitation of emulsion for 6, 12 and 24 hours on a reciprocating shaker (60 cycles per minute) at room temperature. The observations are recorded in Table 10.

Table 9: The effect of centrifugation on different parameters (Sample CsSMES2)

Parameters	Centrifugation speed (rpm)			
	Initial	3000	5000	10000
Phase separation	-	-	-	-
Average particle size (nm)	365± 41.2	366± 55.3	367± 46.7	369± 45.6
Viscosity (mPa-s)	30± 2.45	30± 5.4	29± 3.9	29± 2.6
Zeta potential (mV)	-9.54 ± 0.67	-9.53± 0.34	-9.52± 0.63	-9.50± 0.34
% Entrapment	88.7±3.67%	88.5±3.4%	88.5±1.2%	88.5±3.5%

Note: -   No separation   + Slight Separation

Table 10: The effect of agitation on different parameters (sample CsSMES2)

Parameters	Agitation (60 cycles/min)			
	Initial	6 hrs	12 hrs	24 hrs
Phase separation	-	-	-	-
Average particle size (nm)	365± 41.2	365± 54.3	366± 48.7	367± 46.6
Viscosity (mPa-s)	30± 2.45	30± 3.6	29± 4.3	29± 2.3
Zeta potential (mV)	-9.54 ± 0.67	-9.53± 0.42	-9.52± 0.33	-9.51± 0.45
% Entrapment	88.7±3.67%	88.5±5.3%	88.4±3.2%	88.3±7.5%

[a] Note: -   No separation   + Slight Separation

RESULTS AND DISCUSSION

The mucoadhesive submicron emulsions were prepared by dispersion method using appropriate phase ratio of oil: water (10:90).

Effect of temperature

An increase in temperature decreases interfacial tension as well as viscosity and at the same time it also increases the kinetic energy of droplets and thereby facilitates their coalescence. In the present approach an increase in temperature decreases the particle size and increases the % entrapment of drug as shown in table 1.The optimum temperature was found to be 60°C.

Effect of time of stirring

Time of stirring also exerts a profound and complex influence on the process of emulsification. During the initial period of agitation required for emulsification, droplets are formed; however as agitation continues the chance for collisions between droplets become more frequent and coalescence may occur. In the present approach an increase in time of stirring decreased the particle size and variable results has been obtained for the % entrapment of drug as shown in table 1.The optimum time of stirring was found to be 15min.

Effect of stirring rate

It is quite obvious that the rotation speed of the stirrer affects the particle size distribution and % entrapment of emulsions. As the rotation speed of the stirrer increased from 200 to 400 rpm the particle size decreased and variable results were obtained for % entrapment as shown in table 1. The optimum rotation speed for the system was 300 rpm.

Effect of polymer concentration

Polymer concentration also effects the particle size and % entrapment of the drug by interacting with the drug and the excipients. It also stabilizes the system by interacting with the interfacial tension and it forms a layer over the internal phase of the emulsion. As the polymer (chitosan) concentration was increased from 0.5% to 1.5%, the particle size increased and the % entrapment of drug also increased as shown in table 1. The optimum polymer concentration was found to be 1%

Effect of surfactant concentration

Surfactants decrease the interfacial tension of emulsions. The nature of interaction varies with the physical and chemical behavior of the surfactants. It can also effect the particle size and % entrapment of drug. As the surfactants concentration was increased from 5% to 15%, the particle size increased and the % entrapment of drug

showed variable results as shown in table 1. The optimum surfactant concentration was found to be 10%.

Characterization

Average particle size

The average particle size of optimized formulation was found to be 365nm.Polydispersity index determines the homogeneity of the emulsion. For a homogeneous emulsion it should be <1. It was found to be 0.671 for the optimized formulation as shown in table 3.

Drug content

The drug content of the formulations varied between 72-88.7%. The drug content for the optimized formulation was found to be 88.7% as shown in table 2.

Zeta potential

Zeta potential is a measure of surface charge. It gives an idea about the stability and interaction of charged particles with the mucus layer. For microemulsions with non-ionic surfactants the zeta potential can be used to analyze the charge of the system. Chitosan is cationic in nature and phosphatidylcholine exhibits no net charge at physiological pH levels (Chansiri et al., 1999). The addition of the hydrophilic non-ionic surfactants, Tween 80 to the film led to initial decreases in the zeta potential. Tween 80 may reside on the oil/water interface near the aqueous phase because of their hydrophilicity. This may result in a shielding of the negative surface charge provided by the phospholipids. This phenomenon was not observed with lipophilic non-ionic surfactants. It can be seen that the microemulsion system is negatively charged. The zeta potential of the optimized formulation was found to be -9.54 mV (Table 5).

Viscosity

The slight increment of viscosity might be the result of interaction of submicron emulsions droplets in oil/water systems. It is expected that the hydrophilic chains of Tween 80 are strongly hydrated and connected with hydrogen bonds allowing the interaction between the droplets. The viscosity of optimized formulation was found to be 30 mPas (Table 5). As the polymer concentration increases viscosity also increases.

Mucoadhesive strength %

Mucoadhesive strength depends on the mucoadhesive polymer concentration. It varies between 54-68%. The optimum mucoadhesive % was found to be 65% (Table 5) at polymer concentration of 1%. Interpenetration between the polymeric and the mucosal networks is considered being responsible for the adhesion. As the mucus is negatively charged so the formulation having least negative zeta potential will have highest mucoadhesive %.

In vitro drug release

Release of drug from mucoadhesive submicron emulsion at pH 6.75 in simulated saliva depends upon the entanglement of polymer matrix with the emulsion. The % drug released after 8 and 24 hrs has been presented in Table 4.It has been found that the optimized formulation releases 56.29% of drug after 8 hrs and maximum amount (93.11%) of drug after 24 hrs. It shows the sustained action of the formulation. Statistical data analysis revealed a significant difference between the cumulative amount of drug released of formulations CsSMEt2(1) and CsSMES2(2), CsSMES2(2) and CsSMET3(5), CsSMEt2(1) and CsSMER2(3), CsSMEt2(1) and CsSMEP3(4), CsSMEt2(1) and CsSMET3(5), CsSMER2(3) and CsSMEP3(4) at a probability level of 95% after 24 hr in vitro release study. The difference between 1 and 2 might be due to the difference in surfactant and polymer concentration, the % of drug released being higher at 10% (surfactant) and 1% (polymer). There is marked increase in surface area due to higher polymer and surfactant concentration which leads to more swelling and thus promoted drug release. The difference between 2 and 5 might be due to the difference in surfactant and polymer concentration, the % of drug released being higher at 10% (surfactant) and 1%(polymer).The same reason can be attributed here as above. The

difference between 1 and 3 might be due to the difference in polymer concentration, the % of drug released being higher at 1.5%(polymer).It may be attributed to swelling behavior and an increase in surface area at higher polymer concentration. The difference between 1 and 4 might be due to the difference in stirring rate, the % of drug released being higher at 300rpm. This formula exhibited a higher swelling profile and slower erosion rate. The difference between 1 and 5 might be due to the difference in polymer concentration, the % of drug released being higher at 1.5% (polymer). The difference between 3 and 4 might be due to the difference in surfactant and polymer concentration, the % of drug released being higher at 10% (surfactant) and 1% (polymer). There is comparatively less drug release at 1.5% polymer than 1% as the increase in diffusional path length of the drug may paradoxically delay the release. In addition, the thick gel layer formed on the swollen patch surface is capable of preventing matrix disintegration. Thus it can be concluded that maximum drug release is at the optimized conditions (viz. 10% surfactant, 1% polymer and at 300 rpm).

Drug release kinetics

As depicted by the correlation coefficients between Q and t which is not nearly equal to one shows that it do not follow a linear relationship and so the release kinetics do not follow zero order kinetics. The correlation coefficients between log Q and t also indicate that it did not had a linear relationship and the release kinetics do not follow first order kinetics. It was found that there was a linear relationship between the amount of drug released and the square root of time as the correlation coefficient between Q and $\sqrt{t}$ is closer to1, depending on the state (either dissolved or dispersed) of the drug within the matrix as long as sink conditions applied. So it is concluded that the release kinetics followed Higuchi model showing the release by diffusion process through the polymer matrix.

Mathematically this is represented in the following equations:

$$\text{For a suspension } Q = [D(2A - C_s)C_s t]^{1/2}$$

$$\text{For a solution } Q = 2A \left(\frac{Dt}{\pi}\right)^{1}$$

Or $Q = k_H t^{1/2}$

Where Q is the amount of drug released after time, t per unit exposed area, D is the diffusivity of the drug within the matrix, A is the initial total drug concentration and C_s is the drug solubility within the matrix.

 k_H is the release rate constant, the slope of a plot of Q versus $t^{1/2}$.

Ex vivo permeation studies

The steady state flux (Js) and permeability coefficient (Kp) of the optimized formulation, a dental conventional formulation and an alcoholic solution of metronidazole with the same concentration as the formulation i.e.1% drug concentration are presented in table 6. The results are statistically significant at 95% level of confidence. The steady state flux (Js) and permeability coefficient (Kp) of the optimized formulation has been found to be 1.2 times and 1.46 times greater than the conventional formulation and 46.6 times and 24.6 times greater than the alcoholic solution of metronidazole with the same concentration as the formulation i.e.1% drug concentration respectively. The lag time of the optimized formulation also decreased as compared with the conventional formulation and an alcoholic solution of metronidazole. Thus, it can be concluded that the optimized formulation has higher permeability across the porcine buccal mucosa. The buccal permeability barrier consists of the mucus layer on the surface of the buccal epithelium and the upper layers of the buccal epithelium. The intercellular lipids arising from the membrane coating granules have been implicated as being responsible for this epithelial intercellular permeability barrier. The increased permeability of the formulation may be due to enhancer effect of polysorbates, which could fluidize the lipids of membrane coating granules. Microemulsions solubilise the poorly water soluble

drug and deliver it to the membranes where the drug molecules are released from the microemulsion systems to the buccal epithelial surface, thus increasing the drug epithelial permeation. While, the penetration enhancer effect of lecithin can be mediated by the high affinity of lecithins for epithelium tissue, being able to mix with the lipid components. This behavior can induce a change of the epithelium lipid fluidity, thus leading to an enhanced percutaneous adsorption of drugs.

Antimicrobial susceptibility test

Antimicrobial susceptibility test of the optimized formulation was done by agar dilution method. The inhibition zone diameters are presented in table 7 and shown in Fig 10 and 11. The results indicate that *Porphyromonas gingivalis* is susceptible to both chitosan (1%) and the optimized formulation. The optimized formulation even after 5 times and 10 times dilution showed antimicrobial activity which is above the minimum inhibitory concentration of Metronidazole (MIC of metronidazole is <8µg/ml). The zone of inhibition of optimized formulation at 1:5 dilution was 27.5mm, at 1:10 dilution was 17.4mm and that of chitosan (1%) was 10.6mm.

Stability studies

Effect of temperature

Stability studies was carried out with optimized formulation CsSMES2 which was stored in amber colored glass bottles for a period of 45 days at 4± 1°C, 25± 1°C and 45± 1°C. The particle size of the emulsion was found to increase and viscosity decreased at 45± 1°C, which may be attributed to the coalescence or creaming of the oil droplet at elevated temperature and this is usually coupled with changes in viscosity. There was no effect on phase separation for upto 45 days. The zeta potential and % entrapment of drug slightly decreased after 45 days at all storage temperatures showing slight instability. Non-ionic surfactants have been observed to be sensitive to the changes in the temperature. For example, an increase in the temperature of an o/w microemulsion system prepared with nonionic surfactant can lead to a transition to a w/o system via a bicontinuous structure. In our study there was slight separation at 45± 1°C after 45 days but it was readily recovered after shaking at room temperature.The results of the studies indicated that the formulation was most stable at temperature lower than room temperature.

Effect of centrifugation

The selected formulation was subjected to centrifugation at 3000 rpm, 5000 rpm and 10,000 rpm for 30 min at room temperature. The effect of centrifugation on phase separation, particle size, viscosity, zeta potential and % entrapment of drug was observed. There was no phase separation observed as 10% of surfactant and cosurfactant has stabilized the emulsion by producing an electrostatic barrier which was not disturbed even after high speed of rotation. Viscosity, zeta potential and % entrapment of drug negligibly decreased whereas particle size increased at 5000 and 10,000 rpm. It might be due to the high speed of rotation, agitating the particles and causing coalescence of oil droplets.

Effect of agitation

The effect of agitation was examined by agitating selected formulation for different time periods. As Surfactants can stabilize the emulsion, not only just by forming a mechanical barrier, but also by producing an electrical (electrostatic) barrier or surface charge, so no phase separation was observed at all time periods. The particle size was found to increase slightly and viscosity, zeta potential and % entrapment of drug negligibly decreased after 12 hrs of agitation, which may be attributed to the impingement of droplets upon each other and disorganization of interfacial film and the polymer matrix due to mechanical agitation.

CONCLUSION

The various methods of delivery are beneficial in reducing clinical signs of periodontal inflammation, but these preparations may

cause, staining of the teeth, loss of most of the irrigant solution, inaccurate dosing, may have short duration of action and needs surgical procedure for application or removal.

The aim of the present work was to develop a submicron range mucoadhesive emulsions via utilization of polymer with well defined mucoadhesive features. Beside the obvious advantages of submicron emulsions, including physical stability and ease of preparation, these systems may offer additional benefits for periodontal use. These include an increased drug release rate, due to smaller particle size compared to emulsions, and improved handling due to the transparency of the formulation, the latter making it easy to see the instruments in the working area, i.e. the periodontal pocket. All the formulation excipients taken are non toxic so it is anticipated that the application at the periodontal site is compatible with the patient. The polymer chitosan with mucosal interactive properties will be structurally integrated into the mucosal membrane, so it is anticipated that the drug entrapped within the nano-oil confines or adsorbed at the interface of submicron emulsion are better accomplished to function as a drug carrier as well as retain the delivery system at the mucosal surface for prolonged period of time.

Metronidazole has a spectrum against strictly anaerobic microorganisms and has been used successfully in the treatment of acute necrotizing and ulcerative gingivitis, destructive periodontitis, and chronic periodontitis. In the present approach Metronidazole is being incorporated in mucoadhesive submicron emulsion to give better clinical effects in periodontal diseases and to produce bactericidal levels of the drug in pockets without exposing patients to large systemic doses of metronidazole.

The oil droplets encapsulating metronidazole complexed with lipophilic cosurfactant were successfully dispersed in the aqueous phase containing mucoadhesive polymer and hydrophilic surfactant. The release behavior of encapsulated metronidazole was found to be responsive to the nature and concentration of mucoadhesive polymer and surfactant concentration under the simulated saliva conditions. Ex vivo permeation behavior was also found to be responsive to the nature of surfactants which are acting as permeation enhancers. Antimicrobial studies revealed its action against *Porphyromonas gingivalis* which was contributed to the nature of drug and chitosan.

In the end, it can be concluded that mucoadhesive submicron emulsion of metronidazole is a viable and potential concept for administration into periodontal pocket. Further in vivo studies are necessary to quantify the results and establish its reproducibility at the backdrop of other biological and dietary factors in living beings.

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