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Original Article

5-FLUOROURACIL IMPREGNATED NIOSOMAL-*IN SITU* GEL (THERMO-SENSITIVE) FOR ORAL CANCER: DESIGN, CHARACTERIZATION, *IN VITRO/EX VIVO* EVALUATION

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ABSTRACT

Objective: To formulate and characterize 5-fluorouracil loaded niosomal-*in-situ* gel for sustained drug delivery to reduce dosing frequency at the same time, follow a local drug delivery for cancer targeting.

Methods: Cholesterol and span-60-based niosomes were prepared after following the modified ether injection method. Best formulation selected after characterization through FTIR, SEM, % Entrapment efficiency, zeta potential, polydispersity index, *in vitro* release, and vesicle size whereas, based on cold method niosomes encapsulated in-situ gel was formulated and characterized through gelling temperature and time, spreadability and syringe ability, gel strength, adhesive force, and drug release.

Results: Based on various studies, included particle size, PDI, zeta potential value, % Entrapment efficiency and % drug release, F1 formulation was selected as a best formulation, as niosomal particle size of 388.3 nm proved a higher drug permeation through the buccal area, whereas PDI and zeta potential value of 0.304 and+50.5 are proved a uniform niosomal size with optimum charge distribution which helps to attain higher stability of the formulation, on the other hand % Entrapment efficiency of 87.825% proved that niosomes are capable to hold higher drug concentration; lastly 84.567% of drug release within 12 h of time period prove that higher amount of drug release occur by following sustained release pattern. On the other hand Mucoadhesion, gelling strength and *in vitro* permeation studies prove that niosomes containing in-situ gel has a capacity to adhere over the mucosa with minimum dissolution with saliva up to 12 h and is capable of 95% of drug permeation capacity. Lastly FTIR and SEM images confirmed about niosomal formation with optimum stability.

Conclusion: 5-Fluorouracil encapsulated niosomal in-situ gel will be superior and effective alternative to parenteral dosage forms available in the market for mouth cancer treatment.

Keywords: 5-Fluorouracil, Niosomes, In-situ gel, Span-60, Cholesterol, Modified-ether injection method, Modified-cold method

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INTRODUCTION

Mouth cancer can be defined as an uncontrolled growth of the cells present in any part of the mouth like the buccal, sublingual, pharynx, and even on the lips [1]. Data have proven that cancer is one of the lives inheriting diseases in; which oral cancer is among the list of most common cancers which is responsible for 5% of the total mortalities that happen by the cancer disease over the whole globe [2]. For the treatment of oral cancer, there are many options available in which surgical excision and radiotherapy play a major role but we cannot neglect the role of chemotherapy even though there are a lot of side effects notified during the treatment of cancer in patients, the reason behind these side effects during the treatment of cancer is due to the higher concentration with an improper mode of action over every part of the tissue in body [3]. Nowadays there are a lot of advancements taking place in the case of drug delivery against cancer, like local and target drug delivery which not only have high fluency for target cancer cells even they are modified to reduce the dose-related side effects and toxicity too [4]. The novel drug delivery concept is totally based on how to target such diseases with tackle different challenges that occur during treatment. With the high rate of advantages like reduction of undesired side effects, increase drug stability rate, better storage options with minimum manufacturing cost [5], vesicles, especially niosomes are very common drug delivery systems to target hormonal, vaccine, and cancerous based drugs [6]. Their physiological properties may variate according to the availability of material for preparation and area of the target [7]. They also have an option of flexible structure, which helps to modify drug action over the target area and have a non-ionic structure which is really helpful to enhance drug stability [8]. This is the reason behind the selection of niosomes as local drug delivery in the case of oral cancer. If we target oral cancer by local drug delivery, we find a lot of advantages provided by oral cavity like the hepatic first-pass effect is totally neglected with a high rate of permeability and bioavailability [9], and even dose-related side effects reduce through local drug delivery but there are very less option of novel formulation for local drug delivery against oral cancer because any dosage form can face difficulty during adhering over a buccal area, that is the reason in-situ gels are selected which belong to the list of the dosage form (films, tablets, and patches) have a high rate of adhesiveness, last but not least use of mucoadhesive polymer for preparation of in-situ gel have a great tendency for prolonged the drug release either in a sustained or controlled manner [10, 11], this is the reason behind the selection of Hydroxypropyl methylcellulose and Methylcellulose for preparation of in-situ gel.

MATERIALS AND METHODS

Materials

5-fluorouracil drugs were purchased from Balaji Enterprise, thakordwar society, B/H spinning mill, Varchha road, SURAT-395011(GUJRAT). Span 40, span60, span 80, and Cholesterol were purchased from SD fine chemical limited, Worli Road, Mumbai-400030, (Maharashtra). HPMC, Methylcellulose, Poloxamer 407, Carbopol 934, Cetylpyredinium chloride, Diethyl ether, Methanol was purchased from CDH, Central Drug House (p) Ltd. Daryaganj, New Delhi-110002 (India).

Preliminary study on blank niosomes

To conduct the preliminary study, blank niosomes were prepared through various types of surfactants and methods of preparation, out of which best niosomal preparation was selected on the basis of visual observation through photograph and optical microscopy at 40X [12].

Method of preparation

3^2Full factorial design was followed to prepare niosomes; Ether injection method was followed to prepare niosomes, for which requirement of different concentrations of surfactant, cholesterol and other variants is shown in (table 1). Here in the current procedure Cholesterol, span 60, and cetylpyridinium chloride weighed properly and dissolved inorganic phase containing di-ethyl ether and methanol in a ratio of 8:2. Prepared organic phase solution finally transferred through 22 gauge needle in a beaker containing 20 ml phosphate buffer pH 6.8 and drug solution whose stirring speed of 75rpm and temperature of 65 °C was maintained throughout the time period of 45 min., which is a must require a process to confirm properly shaped vesicles and for high drug loading and also provide an advantage to ensure that organic solution was fully evaporated [12]. In the end, 3^2 full factorial designs utilize to check out the total number of preparation and concentration of independent variables.

Table 1: Formulation design of niosomes as a carrier system based on factorial design (F1–F9)

Formulation code	Drug amount 5-FU (mg)	Dicetylpyridinium chloride (% w/v)	Cholesterol (% w/v)	Span 60 (% w/v)	Ratio of methanol and di-ethyl ether
F1	540	0.2	0.1	0.1	8:2
F2	540	0.2	0.1	1.5	8:2
F3	540	0.2	0.1	3	8:2
F4	540	0.2	1.5	0.1	8:2
F5	540	0.2	1.5	1.5	8:2
F6	540	0.2	1.5	3	8:2
F7	540	0.2	3	0.1	8:2
F8	540	0.2	3	1.5	8:2
F9	540	0.2	3	3	8:2

Characterization of niosomes

Fourier transform infrared spectroscopy

Infrared spectroscopy of 5-fluorouracil, span60, cholesterol, and cetyl pyridinium chloride and their complex was determined with the help of Fourier transform infrared spectroscopy (Shimadzu-8400S, Kyoto, Japan), under a scanning range of 400-4000 cm [13].

Optical microscopy

With the help of visual observation through photomicrograph (HICON, New Delhi, India) at 40x, the prepared formulation examined for confirmation of vesicles formation with its unique structure and shape by using light microscopy [14].

Scanning electron microscopy (SEM)

Images of prepared niosomes were obtained with the help of a Scanning electron microscope (JEOL 5400, Tokyo, Japan). For the proper images, gold was used to prepare a layer over vesicle particles with the help of a sputter coater unit and maintaining an argon atmosphere for proper conductivity, which helps to study about surface morphology of vesicles [12].

Evaluation of niosomes

Vesicle size, polydispersity index and zeta potential

All three studies were conducted with the help of Malvern zetasizer (Malvern Co., U. K.). This was based on the laser diffraction technique done for light scattering. Here temperature was maintained at 25 °C. Vesicle size, zeta potential, and polydispersity index of all prepared niosomal formulations were determined by Malvern Zetasizer (Malvern Co., UK), which is based on light scattering based laser diffraction technique. To conduct the following technique, samples were sonicated for 15 min and then the sample was diluted with the help of deionized water and finally filtered through a membrane having a pore size of 0.45 µm for particle size analysis and polydispersity index. The same procedure was performed without dilution for charge determination of vesicles at 25 °C [15].

% Entrapment efficiency

Entrapment efficiency for all the vesicles was calculated with the help of the centrifugation method in which the required amount of niosomal preparation was filled and centrifuged at 20000 rpm for 40 min at 4^0 C. After centrifugation, a blurred supernatant solution was analyzed with the help of a UV spectrophotometer at 266 nm. Finally, the amount of drug entrapped was calculated with the help of the formula given below [16, 17].

$$EE\ \% = \frac{\text{Total amount of drug} - \text{amount of unbound drug}}{\text{Total amount of drug}} X\ 100$$

***In vitro* drug release**

A drug release study of niosomes was performed with the help of the dialysis bag method. To conduct the current study 10 ml of niosomal suspension was placed in cellophane membrane, which was first dipped for 24 h in phosphate buffer media and then tied up from both sides after filling 10 ml of niosomal sample, then dipped in 500 ml of phosphate buffer pH 6.8 and stirred at 50rpm with the help of magnetic stirrer at a temperature of 37°±2 °C. Then samples were withdrawn at a different time interval to analyzed drug concentration at 266 nm, at the same time, DD Solver software was use to cross-check the end result of drug release [18].

Release kinetics studies

To understand the behavior of drug release at giving time period, first calculate the release kinetics in which we had to calculate the different kinetic equation formula or equation, all these kind of data are included in software named BIT SOFT version1.12. This release kinetics software is quite useful in case when we require accuracy in less time duration and also withstand for various orders and models including zero order, first order, Higuchi's model and Peppas model. At the same time there is no requirement of preparation of graph in-between % cumulative drug release vs. Time, log value of % drug remaining vs. Time etc, the only thing is needed to add is drug release data to check out the actual order of drug release. [19–21]

Selection of optimized formulation

Best formulation was selected on the basis of giving parameters that were performed for the niosomes, like % CDR, % EE, % Yield, Vesicle Size, PDI, and Zeta potential value.

Preparation of niosomes containing *in situ* gel

Based on the cold method *in situ* gel was prepared, in which weighed amount of poloxamer-407 and fixed amount of poloxamer-188 transfer into a beaker containing 60 ml of distilled water with fixed temperature (4 °C) and stirring speed (500 rpm) maintained with the help of magnetic stirrer for 2 h of the time period. Finally, at 4±2 °C, the solution was kept for 12 h in the refrigerator. On the other hand, a pre-weighed amount of HPMC/MC/carbopol-934 P with a fixed amount of niosomes containing the drug were added to above mention solution of poloxamer during continuous final stirring for 45 min [22, 23], which provide an *in-situ* gel in sol form, whose composition mention in (table 2).

Table 2: Formulation designs of niosomes loaded *in situ* gel formulation as carrier system (NIG1–NIG6)

Formulation code	P-407 (gm)	P-188 (gm)	C-934P (gm)	MC (gm)	HPMC (gm)	5-FU (mg)	Niosomes (%w/v)
NIG1	14	2	1	--	--	540	10.3
NIG2	18	2	--	0.5	--	540	10.3
NIG3	14	2	--	--	0.5	540	10.3
NIG4	18	2	--	--	1	540	10.3
NIG5	14	2	--	1	--	540	10.3
NIG6	18	2	2	--	--	540	10.3

Validation of niosomes loaded *in situ* gel

Clarity and drug content

All six preparation of in situ gel was tested for an appearance with clarity which was observed and recorded in presence of white light and against black background just by visual appearance. Whereas drug content was measured after dilution of gel with phosphate buffer pH 6.8 as a media and analyzed the drug content was after notifying the absorbance against phosphate buffer as a blank media. All reading was performed in triplicate [24-26].

pH, gelling temperature and gelling time

The pH of the sample was measured with the help of a digital pH meter. Whereas gelling temperature and time were measured after the solution was placed in a test tube and dipped in a water bath, whose temperature is already maintained at 37 °C±2 °C. The time and temperature of the converted gel were finally noticed with the help of a thermometer and stopwatch. All three data readings were performed in triplicate and expressed with mean value (n=3) [26-28].

Spreadability and syringe ability

For measurement of spreadability, first transfer 0.5 grams of gel over a clean glass slide and then with the help of another glass slide, covered it gently by applying 1000 grams of weighed over it, finally remove the weight after one minute time period and measured the total diameter formed after spreading of the gel, which helps in the determination of spreadability of gel. Results represent after calculating the data in triplicate and expressed with a mean value (n=3). In the second case, calculation of syringeability of all formulations of *in-situ* gel, first of all, transfer 2 gram of gel into a beaker and sucked the gel with the help of 20 gauge needle containing syringe. Based on the result, those gel passes too quickly from the syringe are included as a pass (P). Whereas those gels faces difficulty in passing through syringeability are included as fail (F) [29].

Viscosity

The rheological-based characteristics of niosomal containing in-situ gel sample was measured with the help of a T-96 spindle containing Brookfield optical viscometer (Brookfield Engineering Laboratories, Inc, MA). By transferring 50 grams of gel in a container and dipping the spindle into it, where rotation occurs at various angular velocities at the temperature of 25 °C. Results were represented after calculating the data in triplicate and expressed with mean value (n=3) [30].

Gel strength and adhesive force

Gel strength was measured by transferring a 25 g of sol in a 100 ml measuring cylinder and dipping it in a water bath to convert it into a gel form; then, the gel was transferred in a 500 ml of beaker where 50 g of weight over the measuring cylinder is applied and noted down the time required to sink 5 cm down of measuring cylinder in a gel. Whereas in case second, a study was conducted to calculate the adhesive force required to separate the attached gel over the buccal mucosa, which was proof of the mucoadhesive force of gel and also inform about the strength of gel which actually matches with the full drug release time period of 12 h or not. To conduct *in vitro* mucoadhesion, an instrument was utilized to measure the mucoadhesive force, which is given below in (fig. 1(a)**)** and first explained by Qi and his coworkers in 2007 [31]. To conduct this study, the obtained goat buccal mucosa was washed with a buffer solution of pH 7.4. Finally, the mucosa piece was dipped in the ringer salt solution for at least 30 min. Then, the buccal piece (G) was attached over pan balance, and then the gel was placed in the sample holder (f), which was arranged with a water bath (E). In the end, added the weight over (B) to confirm the actual contact between the gel and buccal mucosa. Finally, water drop was added drop-by-drop in a beaker which was arranged to the right pan (C), and water was running through burette (I). The stress was measured over the buccal mucosa by applying the formula given below [32, 33].

$$\text{Detachment stress } = m \times \frac{g}{A}$$

Where

m= weight on balance

g = acceleration force due to gravity.

A = tissue area over gel applied

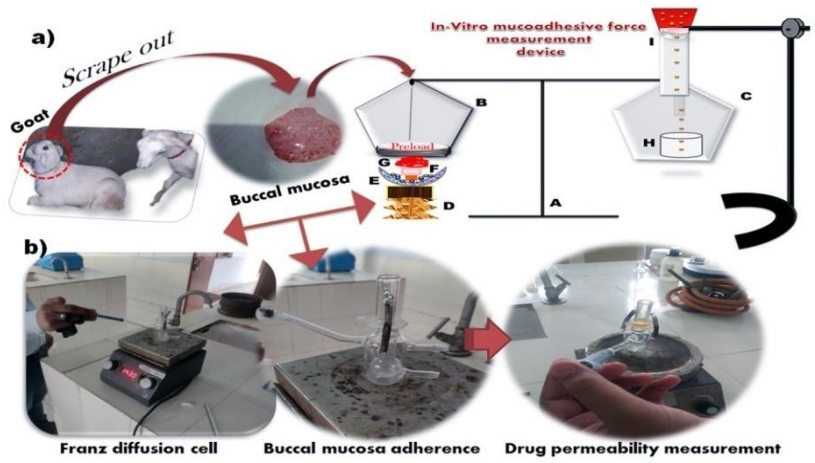

Fig. 1: Diagrammatic representation of mucoadhesive force through assembly arrangement and, fig. 1(b) Experimental setup of Franz diffusion cell and drug permeability measurement

In vitro drug release

Niosomal *in-situ* gel drug release was performed with the help of the dialysis bag method. To check out drug release through dialysis bag method, cellophane membrane was required, which was first dipped in the phosphate buffer pH 6.8 overnight and then in-situ gel sample was filled and tied up from both sides and dipped in 500 ml of media (phosphate buffer pH 6.8) with maintaining stirring speed at 50rpm with the help of magnetic stirrer at a temperature of 37 °C±2 °C. Then 5 ml of sample was withdrawn at different time intervals and, the media was replaced with fresh buffer solution. Finally, samples were analyzed at 266 nm at the same time DD Solver software was use to cross-check the end result of drug release [34, 35].

Selection of optimized formulation

All in-situ gel formation was optimized on the basis of the drug content, gel strength, spreadability, mucoadhesive force, *in vitro* drug release, and lowest gelling temperature and gelling time with optimum gelling pH. Selected formulations were ready for further testing procedures.

Characterization of optimized niosomal-*in situ* gel

Ex-vivo permeation study

Ex-vivo permeation study conducted with the help of Franz diffusion cell, shown in (fig. 1. b, in which excised layer of goat buccal mucosa was used for permeation of gel before which mucosa was first cleaned and dipped in phosphate buffer solution (pH 6.8). Phosphate buffer media with magnetic bead was present in the receptor compartment and 0.5 grams of gel was present in the donor compartment. Whereas the donor and the receptor compartments separated by the suitable size of the buccal mucosa. Finally, the sample was withdrawn at various time intervals and analyzed for drug permeation with UV spectroscopy at 266 nm. Results represent after calculating the data in triplicate and expressed with mean value (n=3) [34, 36].

Comparative *in vitro* release analysis

On the basis of in situ drug release study, final comparison occurred with the help of dialysis bag method in which different samples of Flonida 5% cream (marketed preparation), best optimized niosomal formulation (F1), and best optimized in-situ gel (NIG-1) was analyzed. To conduct the current procedure, all formulations were filled in the dialysis bag separately and dipped in the 250 ml of phosphate buffer pH 6.8, which was used as a media by maintaining the stirring speed of 100rpm and temperature of 37±2 °C with the help of a magnetic stirrer to mimic the buccal environment, lastly collect the 2 ml of samples at different intervals of time (0,1,2,3,4,6,8,10,12) in hrs for drug release comparison.

RESULTS AND DISCUSSION

Preliminary study

On the basis of various surfactants and method of preparation blank niosomes was prepared and the best formulation containing vesicles were selected on the basis of photo-micrographic results shown in (fig. 2). Which is divided into two different cases, In case I where blank niosomes in the presence of cholesterol and different surfactant at a ratio of 1:1 were formed, out of which niosomes containing span 60 showed the best shape and uniform size distribution, whereas in images of span 20 and span 40 there is no niosomes were observed and in case of span 80, niosomes were formed but had an undefined and non-uniform structure. In case II where blank niosomes in presence of cholesterol and span 60 at a ratio of 1:1 with different methods of preparation were formed, out of which ether injection method was selected because this method again showed a uniform size distribution of vesicles and had the tiniest vesicle size out of all other preparation, whereas in case of sonication and handshaking method very few numbers of niosomes formed with a large amount of free surfactant is observed and lastly in thin-film hydration method very large and irregular shape vesicles were observed.

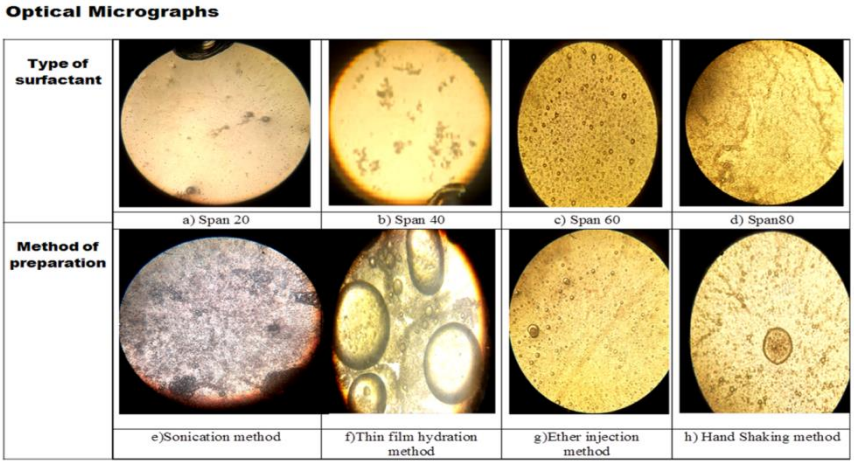

Fig. 2: Showing photomicrographic images of niosomes based on various surfactants and method of preparation

Characterization of niosomes

FTIR

FTIR spectra were used to find if any interaction occurred between drug and excipients because of which an unstable formulation occurred and cause a harmful impact on the body. Here FTIR data of excipients, drug, and their complex are shown in (fig. 3), including the 5-FU, Span-60, cholesterol, cetylpyridinium chloride, and their complex, all have their characteristics peaks in the range of 4000-400 cm^{1}. The 5-Fluorouracil drug was shown a characteristic peak at 3999 cm-1 was due to stretching of–NH group and peak at 3050 cm^{-1} was due to C-H stretching vibration and further another characteristic peak was noticed at 1720 cm^{-1}, 1655 cm-1, and 1649 cm^{-1} showing a wide-stretching because of the presence of single group C=O, and the last peak was noticed at 1246 cm^{-1} because of the presence of C-F group. On another hand cetylpyridinium chloride shows characteristics peaks at 3468 cm^{-1} formed due to bending of N-H group, 2950 cm^{-1} due to presence of–OH group, 1645 cm^{-1} due to presence of C=N group and 1421 cm^{-1} was due to presence of C-F group; similarly spectrum of span and cholesterol shows characteristics peaks, which was observed in FTIR at 3250 cm-1, 2917 cm-1, 1736 cm-1and 1490 cm-1 indicate the presence of absorption bands due to C-O group, C-H group, stretching of C=O and bending of N-H groups. In last FTIR spectra of blend formed after continuous mixing of all excipients with the drug, in which different spectral peaks were observed at 3496 cm^{-1}, 2915 cm^{-1}, which was formed due to the presence of N-H and O-H group and prove the presence of cetylpyridinium chloride whereas peaks observed at, 1741

$cm^{-1}$and 1679 cm^{-1} formed due to stretching of C=O group and peak at 1246 cm^{-1} formed due to C-F bonding and all three spectral peaks prove the presence of span 60 whereas spectral peak observed at 2848 cm^{-1}proved the presence of cholesterol. After observation of all peaks present in a blend named complex R, it is proved that all peaks were observed at their respected area with a minor adjustment which is the only proof that the formation of the blend occurred without any interaction between drug and excipients.

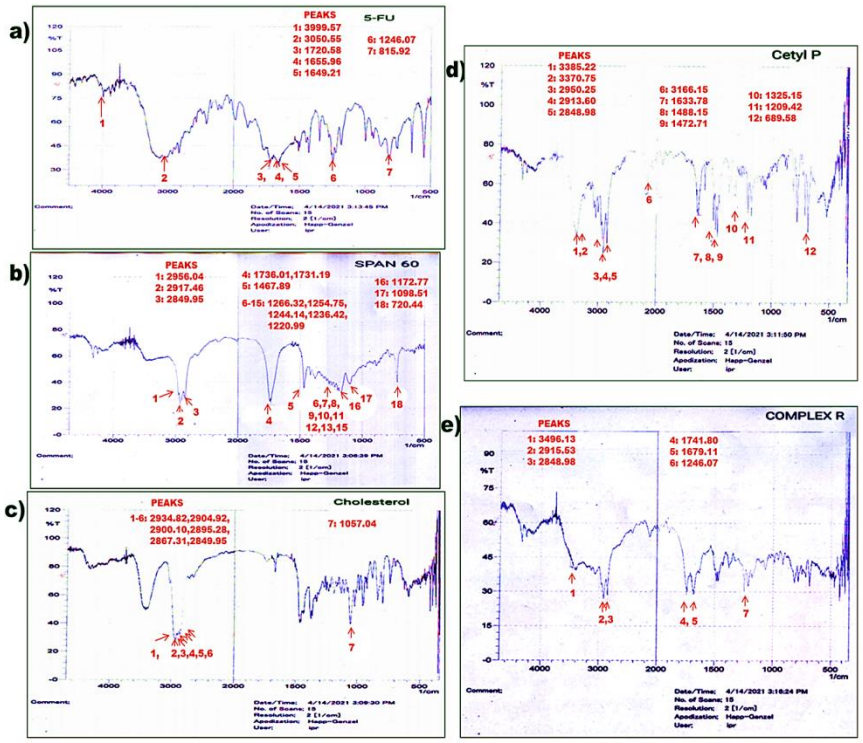

Fig. 3: Showing an FTIR images of (a) 5-fluorouracil, (b) Span 60, (c) Cholesterol, (d) Cetylpyridinium chloride and their (e) Complex

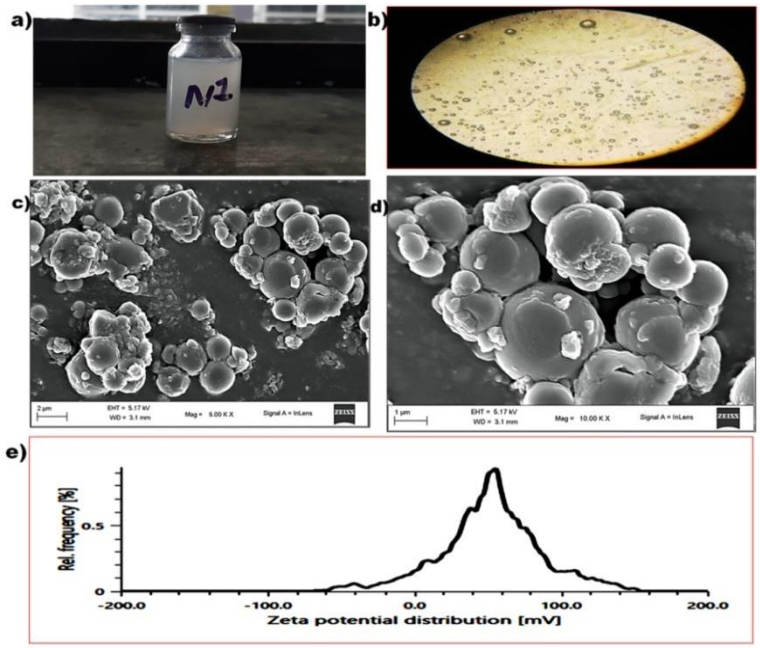

Fig. 4: Showing an (a) simple photographic image of niosomal formulation F1 (b) Optical microscopy based image of niosomes formed in presence of span 60, (c) Span 60 based SEM photographs of niosomes at 1 µm and (d) at 2 µm and (e) Zeta charge value over the niosomes

Optical microscopy

Optical microscopy generally helps to confirm the formation of vesicles and inform a little bit about the structure and shape-related phenomenon of vesicles, before observing optical microscopy, a simple photographic image of niosomal formulation observed which is shown in fig. 4(a). After seeing this image it is clear that niosomal formulation is of blurred appearance which is proof of uniform

distribution of niosomes throughout the formulation, whereas at bottom of the container highly dense suspension type appearance notice this may be because of the large size vesicles settle down at bottom of the container. On another hand, a photomicroscopic image of niosomes was observed and shown in fig. 4(b). Where a clear view of hollow niosomes which formed in the presence of cholesterol, span 60, and cetylpyridinium chloride were observed. Here photomicrographic image just confirmed the formation of niosomes with proper spherical shape [37] and was noticed as single layer vesicles without any agglomeration.

Scanning electron microscopy

SEM is generally performed to study about vesicle's size, shape, structure, and other morphological behavior observed in images. With the help of (fig. 4(c)) and (fig. 4(d)), it is confirmed that niosomes were formed. In fig. 4(c) it is clearly observed that all niosomes have a uniform spherical shape with a similar size range because of which a low PDI value is observed. Whereas fig. 4(d) confirmed that niosomes are unable to maintain a uniform distance with each other, because of which a high zeta potential value (beyond 50 mv) is observed, whose image mentioned in (fig. 4(e)).

Evaluation of niosomes

Particle size

Result based on mean particle size shown in table 3, which conclude that when we increase the concentration of span 60, the size of the vesicle increased this is due to the fact that surface free energy increase because of decreased hydrophobic behavior, which is one of the major cause of size increment in vesicles. Whereas when the amount of cholesterol increases the average particle size of formulation F1-F3, F4-F6 and F7-F9 were decrease due to the fact that at higher concentration, cholesterol tries to enter in the bilayer of vesicles where it increases the order of chain reaction due to the reason close packing of vesicle occurred [38].

Polydispersity index (PDI)

PDI value helps to notice homogeneous behavior of the distribution of particles as well as their uniformity of size. PDI values range from 0 to 1 and value shifting towards zero is proof that higher and uniform particles distribution. Here PDI study for all the formulation (F1to F9) is shown in table 3, through which it was noticed that when we enhance surfactant concentration, it definitely help to provide the smaller PDI value range [36] of 0.214-0.304, which signifies that all formulation have uniform size distribution and have a minor change in a size range of niosomes which is acceptable for buccal route penetration and formulation also notice without any aggregation [40].

Zeta potential

Zeta potential helps to notice the particle surface charge. This indicates the degree of repulsion and stability of the formulation. After seeing zeta potential data in table 3, which further conclude that, zeta potential value for the niosomal preparation found in the range of+50.5 to+68.0 due to the presence of cetylpyridinium chloride which provides a positive charge because of its hydrophilic Cationic behavior. On the basis of niosomal preparation result which is not belong to the zeta potential stability system range (+30 to-30), this can be concluded that niosomal formulations are not stable enough for a longer period of time, and after a few months aggregation may occur in between the vesicles [41].

% Entrapment efficiency

The reason behind cholesterol used in the preparation of niosomes is that they provide stability to the structure of niosomes and have cement leaking quality, both of these qualities are concentration-dependent; beyond optimum concentration cholesterol shows a negative impact over niosomes structure as well as drug concentration which is observed here in this study. Entrapment efficiency was performed with the help of the centrifugation method and the range obtained for % entrapment efficiency was between 34.33 to 88.665which is shown in table 3. Here the three different concentrations was used in preparation of niosomes like 0.1%w/v for F1-F3, 1.5% w/v for F4-F6 and 3% w/v for F7-F9. Out of all three 0.1 % shows highest drug entrapment (88.665%) because cholesterol concentration was optimum to hold drug inside niosomes and provide cement leaking property whereas when we move towards 1.5% and 3% of concentration highest drug entrapped was only 56% and 36% this is because cholesterol cross it's optimum concentration level and now it competes with the drug to entrapped itself or packs itself in the empty space of the bilayer of the vesicle, because of this drug entrapment decrease when the concentration increase beyond the limit [42].

Cumulative drug release

% Drug release data are shown in (fig. 5) is based on the dialysis bag method in which the diffusion rate of the drug was calculated. In vitro drug release range for all niosomal formulations was found within the limit of 30.138 to 84.567 % after 12 h of drug release, observed in (table 3) which totally depends on the concentration of cholesterol and the type of span used for niosomes preparation. In case first, when we increase the concentration of cholesterol, entrapment efficiency of niosomes decreased as less concentration of drug hold by niosomes because of which less drug release occurred, all this happen because beyond the optimum concentration of cholesterol inside the vesicle, it competes with the drug concentration and reduces drug entrapment efficiency of niosomes, whereas in case second, when we reduce the concentration of cholesterol we found a higher entrapment efficiency of the drug by niosomes as a lower concentration of cholesterol help in the reduction of membrane permeability which directly reduces the drug lost from vesicle and increase the tendency of entrapment of drug which finally results into an enhancement of drug release [43]. We noticed a constant 12 h drug release of drug through niosomes, which is only possible due to the presence of span 60, as span 60 presents with the longest alkyl chain, which slows down the drug release through vesicles and helps to achieve a constant drug release up to 12 h of the time period [37].

Table 3: Evaluation parameters of 5-fluorouracil loaded niosomes

Formulation code	*Vesicle size (nm)	*Zeta potential	*Polydispersity index	*% Entrapment efficiency	*%CDR
F1	388.3±0.2	50.5±0.22	0.304±0.32	87.825±0.06	84.567±0.08
F2	782.4±0.05	53.2±0.81	0.29±0.34	88.665±0.06	83.640±0.1
F3	1180.2±0.2	57.3±0.42	0.214±0.57	85.648±0.2	81.017±0.04
F4	323.1±0.5	56.1±1	0.292±0.04	56.16±0.5	51.295±0.05
F5	652.4±0.6	54.3±0.98	0.231±0.08	53.806±0.08	49.690±0.02
F6	824.2±0.32	61±1	0.24±0.1	49.33±0.04	42.592±0.12
F7	351±0.57	58.6±2	0.275±0.82	34.842±0.3	30.138±0.2
F8	602.4±0.86	63.6±0.24	0.264±0.86	37.842±0.4	33.811±0.1
F9	637.2±0.56	68±0.92	0.251±0.2	37.002±0.8	36.805±0.08

*Each value is the average of three experiments±SD

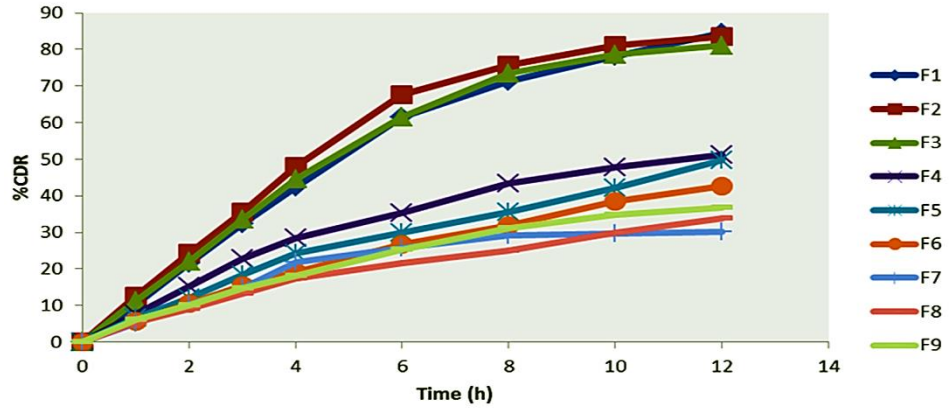

Fig. 5: *In vitro* release profile(s) of formulation(s) F1-F9, drug release based values are given as mean; where n=3

Release kinetics studies

To understand the release kinetics of niosomal formulation in phosphate buffer media, all four different models' R^2 values were calculated on the basis of the graph in-between %CDR and time in a different manner. The result suggested that most of the formulation follows first-order drug release mechanism which is proving that drug release depends on the concentration of drug in vesicles as we find at the beginning of drug release higher concentration was present inside the vesicle because of which higher drug release rate noticed whereas in later hours of drug release when the concentration of drug in vesicle decrease at the same time % drug release was also reduced. Also in formulation F1-F3 having higher drug loading release a higher amount of drug in a given period of time whereas niosomal formulation F7-F9 having very less drug loading because of which low amount of drug release were notice based on concentration which was further decreased with respect to time. A calculated regression analysis provided the linear curve with R^2 value is shown in (table 4), given below [44].

Table 4: Evaluation of release kinetics of niosomes formulation(s)

Formulation name	Zero order (R^2)	First order (R^2)	Higuchi plot (R^2)	Peppas plot (R^2)	Best-Fit model
F1	0.948	0.997	0.964	0.751	First order
F2	0.916	0.983	0.962	0.714	First order
F3	0.931	0.985	0.963	0.734	First order
F4	0.943	0.979	0.979	0.755	First order
F5	0.976	0.992	0.968	0.790	First order
F6	0.981	0.996	0.966	0.816	First order
F7	0.853	0.874	0.948	0.774	Higuchi plot
F8	0.967	0.984	0.979	0.797	First order
F9	0.961	0.979	0.973	0.792	First order

Selection of optimized formulation

On the basis of %CDR, % Entrapment efficiency, and PDI result, formulation F1 was selected as the best formulation as it is able to show maximum desirability as a niosomal formulation. Result of percent cumulative drug release (84.567±2.11 %) which is highest among all formulations and % entrapment efficiency (87.825±1.81) with an acceptable particle size range of (388.3±3.05 nm) and optimum PDI range (0.304±5.51), which is proving that formulation F1 is one of the best formulations among all and ready for further comparison with marketed preparation.

Evaluation of *in situ* gel

Clarity and drug content

All prepared *in-situ* gel was tested for their appearance on the basis of clarity level and for drug content. A clarity test is proof of no residue found in the formulation because of which reduction in the chance of itching and irritation type of effects over the body. After seeing the result based on clarity testing in table 05, it is observed that the first five formulations (NIG1-NIG5) are fully transparent and clear with a smooth texture which means that the amount of polymer used for the preparation of gels are fully dissolved whereas in case of NIG6 formulation found with less clarity and this is a proof of an undissolved residue of polymer left during gel formation or higher amount of polymer may be used for gel formulation because of which residue of polymer left and cause the less transparent appearance of a gel. On the basis of the result shown in table 05, the drug content of all six formulations was noticed in the range of 82.63±0.14 to 86.3±0.32. Result proof that one of the best methods utilized for in situ gel preparation as there was negligible drug loss observed and the exact amount of niosomes was incorporated inside *in-situ* gel which helps to give an acceptable range of drug content.

pH, gelling temperature and gelling time

Gelling pH of all formulation was lies in between the range of 5.9±0.2 to 6.9±0.0 shown in (table 5), proving that all formulation, except NIG-1, matches the pH of the buccal area because of which patient did not face any challenge or discomfort during its use. The gelling temperature of all formulations was noticed below the body temperature which again proves that all formulations are easily converted to sol form during body contact and did not face any challenge in sol to gel conversion, in last gelling time range was noticed in between 26.6±8.6 to 37.8±2.4 shown in table 5, which means all formulation convert to gel nearby 30 sec, this time period will reduce the chance of contact in between sol with saliva and finally support the higher gelling strength and mucoadhesive power of gel.

Spreadibility and syringeability

The phenomenon of Spreadability is inversely proportional to the viscosity of the gel. The concentration of the polymer used in the preparation of the gel rose at the same time Spreadability of the gel was reduced which is quite noticeable in the given (table **6**). Here maximum spreadability is shown by NIG1 formulation (6.86±0.02), which had a minimum concentration of poloxamer-407, poloxamer-188, and carbopol 934P because of which a loose bond in between particles of the polymer occurs and gel formed by these two

polymers is able to spread wide apart after applying pressure, whereas NIG4 contains the highest concentration of polaxamer407, poloxamer-188, and HPMC, which further help to form and maintain a tight grip with polymers because of which a minimum Spreadability noticed. Whereas in case second of syringeability, again the result relates to the viscosity of the sol, as syringeability is inversely proportional to the viscosity when a higher concentration of polymer used highly viscous sol form occurred because of which difficulty faced by the sol to pass through the syringe, the result is shown in table 6, proved that last three preparation (NIG-4, NIG-5, and NIG6) formed in presence of HPMC, MC and carbopol-934 P, contain the highest concentration of all three polymers because of which all three formulation faces difficulty to pass through the syringe.

Table 5: Evaluation data 1 of niosomes loaded *in-situ* gel

Formulation code	*Gelling temperature (%w/v)	*Gelling time (seconds)	*pH of sol	*pH of gel	*%Drug content
NIG1	30.4±0.083	26.6±8.6	5.9±0.2	5.7±0.2	84.02±0.58
NIG2	32.6±0.098	32.3±3.4	6.1±0	5.4±0.2	86.23±0.3
NIG3	32.9±0.763	35.8±2.3	6.5±0	5.6±1	84.29±0.97
NIG4	35.0±0.26	33.4±1.8	6.9±0	6.1±0.8	86.3±0.32
NIG5	33.8±0.69	37.8±2.4	6.6±0.2	5.4±0.2	85.14±0.44
NIG6	37.1±0.06	31.1±3	6.8±0.2	5.7±0.4	82.63±0.14

*Each value is the average of three experiments±SD

Table 6: Evaluation data 2 of niosomes loaded *in-situ* gel

Formulation code	*Spreadibility	Syringeability	Gelling capacity	Clarity
NIG1	6.86±0.02	P	+	T
NIG2	6.54±0.08	P	++	T
NIG3	6.24±0.24	P	++	T
NIG4	5.18±0.26	P	+++	T
NIG5	5.42±0.08	P	+++	T
NIG6	5.86±0.05	p	+++	L

*Each value is the average of three experiments±SD

T-Transparent, L–less transparent

+Gelation but dissolve slowly, ++Gelation and remains for few hours, Gelation and remains for many hours. P–Pass, F-Fail

Viscosity

Viscosity is among the most critical factors to calculate during the determination of the buccal residential time period of the desired formulation. The viscosity of all six formulations is shown in (fig. **6**), which is proof of actual variation occurring in viscosity data of all *in-situ* gels in the presence and absence of phosphate buffer media. This confirms that all formulation is a true example of the pseudo-plastic flow which means in presence of liquid a decrease of viscosity of gel occurs or can say that shear-thinning rheological behavior is noticed. the data proof that all three different types of gel formed in presence of carbopol 934P, MC, and HPMC had a capacity to produce gel in presence of polaxamer through the cold method and all improved the viscosity remarkably because of the reason that all have a minor or major capability to produce gel in presence of mono or divalent cations available in phosphate buffers media but HPMC has the highest capability to reproduce gel in presence of phosphate buffer that is the reason why formulation NIG-4 is able to show the highest viscosity of 5679 cps in absence of phosphate buffer as a media and 2953cps in presence of media, where others are not [45-47].

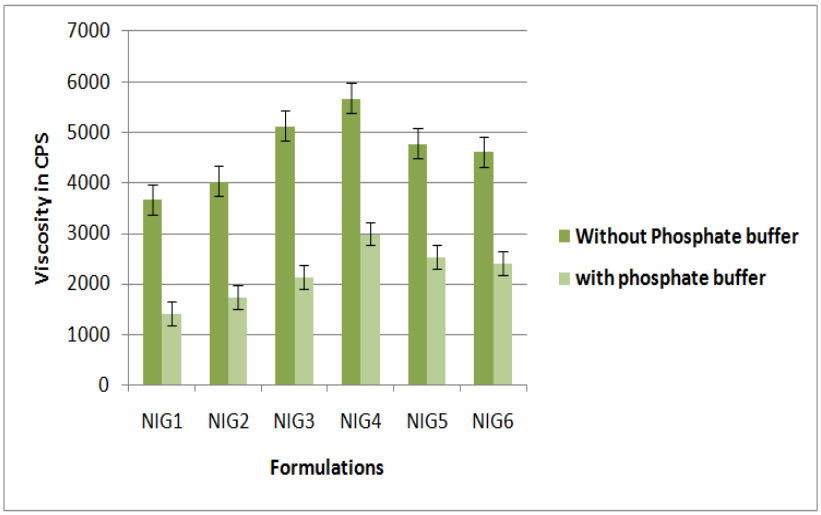

Fig. 6: Rheological behavior of different polymer based *in-situ* gels (NIG1-NIG6) in presence and absence of phosphate buffer pH 6.8, all graph based values are given as mean±SD; where n=3

Gel strength and adhesive force

Gel strength and adhesive force are the two factors that help to determine the retention time of gel over mucus membrane, method explained by Qi helped to calculate the adhesive force and gel strength whose end result is shown in (table 7). Which proof that gel strength and adhesive force factors are directly proportional to the viscosity of gels, and when the concentration of all three polymers

rises their gelling capability and mucoadhesive nature are also enhanced, the reason behind this phenomenon is that when gel comes in contact with buffer solution they utilize the electrolytes to enhance gelling power present in a buffer because of which their actual gelling strength and mucoadhesive force increased and among all six formulations, NIG4 shows highest gel strength 155±0.06 g and adhesive force 5.9±0.2 N mm. This definitely proves that among all three different polymers HPMC has the highest tendency to combine with free electrolytes than the other two polymers, that's why in presence of phosphate buffer media HPMC containing *in-situ* gel shows the highest gel strength and adhesive force [45-47].

Table 7: Evaluation data 3 of niosomes loaded *in-situ* gel

Formulation code	*Viscosity (cP)	*Gel strength (g)	*Adhesive force (N mm)
NIG1	3665±0.21	104±0.03	4.3±0.2
NIG2	4028±0.09	129±0.09	4.7±0.1
NIG3	4427±0.32	132±0.13	5.0±0.1
NIG4	5679±0.36	155±0.06	5.9±0.2
NIG5	4785±0.07	140±0.23	5.4±0.2
NIG6	4714±0.05	137±0.17	5.6±0.4

*Each value is the average of three experiments±SD

Drug release

Actual data of drug release of *in-situ* gel containing niosomal encapsulated 5-fluorouracil drug shown to check the actual drug response after absorption of the drug through buccal route. Comparison between various drug release profiles of the *in-situ* gel is shown in (fig. 7). Which is proof of variation of drug release and this variation depends on polymers and their concentration used in the preparation of in-situ gels. The highest and lowest drug release after 12 h of the time period was noticed in formulation NIG1 (83.367 %) and NIG4 (61.384) and all this happen because NIG1 contain a minimum concentration of carbopol 934 P because of which a less viscous gel occur with weak intermolecular bonding in between carbopol molecules, this bonding becomes weak in presence of phosphate buffer as a media and finally help in to lose strength because of which drug release through gel attain a faster process whereas in case of NIG4, which have the highest concentration of HPMC polymer which make a hard intermolecular bonding and have a property to reform the gelling network even after the separation of molecules in presence of phosphate buffer as a media because of which HPMC shows a higher gel strength and cause a controlled and slow drug release.

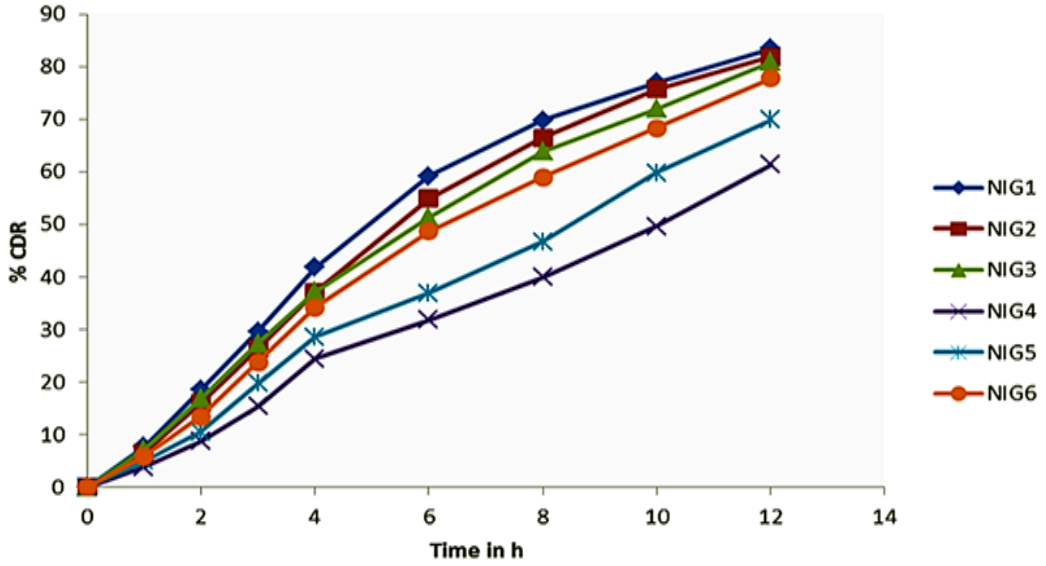

Fig.7: *In vitro* release profile(s) of formulation(s) NIG1–NIG6, drug release based values are given as mean; where n=3

Selection of optimized formulation

On the basis of drug content, gel strength, Spreadability, mucoadhesive force, *in vitro* drug release, lowest gelling temperature, and gelling time with optimum gelling pH, NIG3 and NIG6 were Selected as the two best formulations, which was ready for drug permeation study to select overall best formulation.

Characterization of optimized niosomal-*In-situ* gel

***Ex-vivo* drug permeation study**

On the basis of characterization of *in-situ* gels, NIG-3 and NIG-6 was selected as the best *in-situ* gel formulation and evaluated for drug permeation studies, in which it was found to be 77.86 % and 73.68 % of drug permeation, occurred by NIG-3 and NIG-6 formulation after 12 h of the time period shown in (fig. 8), high amount of drug permeation occurred by *in-situ* gel, which only happens because of encapsulation of niosomes of size 388.3 nm in an *in-situ* gel. It is clarified by Teubl *et al.*, 2013 and Hua *et al.*, 2019 [48, 49], in their studies that the rate of drug permeation is enhanced in a case when vesicle size occurs nearby 300 nm. On another hand, 100 % of drug permeation does not occur because of the reason that *in-situ* gel formed by HPMC may start to dissolve in saliva after a few hours because of which less drug permeation occurs but too hard intermolecular bonding even in presence of saliva nearby 90% of drug permeation was observed.

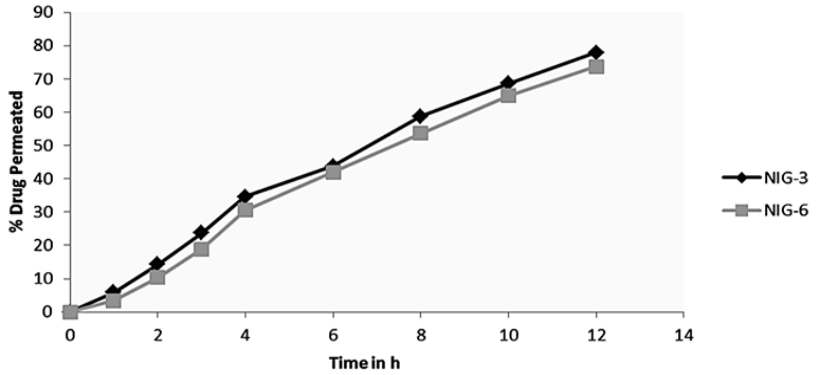

Fig. 8: *In vitro* release profile(s) of formulation(s) NIG1–NIG6, drug release based values are given as mean; where n=3

Table 8: Percent drug release comparison in between F1, NIG3 and Flonida-5%

Time (h)	*F1	*NIG3	*FLONIDA 5%
0	0	0	0
1	10.292±0.03	7.141±0.26	69.484±0.26
2	21.619±0.01	16.839±0.15	85.407±0.31
3	32.36±0.21	27.352±0.5	88.183±0.4
4	42.437±0.47	37.157±0.76	88.183±0.09
6	61.404±0.04	51.372±0.36	88.183±0.37
8	71.296±0.01	63.897±0.42	88.183±0.09
10	78.24±0.65	71.98±0.43	88.183±0.02
12	84.567±0.08	80.891±0.76	88.183±0.37

*Each value is the average of three experiments±SD

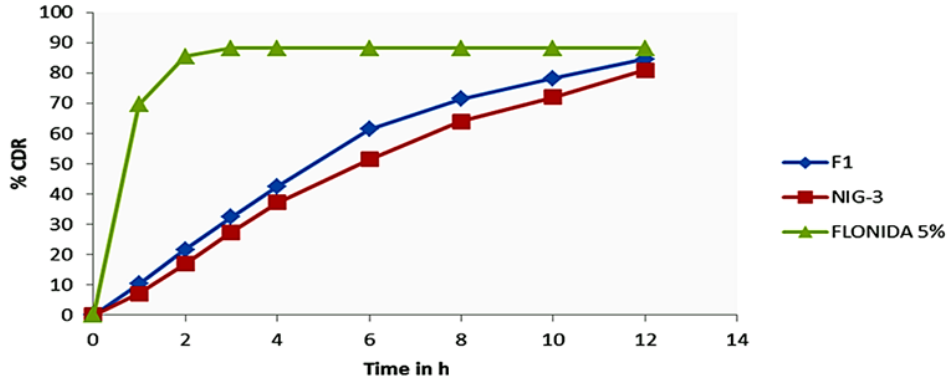

Fig. 9: Drug release profile of F1, NIG3 and marketed formulation (Flonida®-5%)

Comparative *in vitro* drug release study

Drug release comparison in between three different formulations, out of which F1 and NIG-3 are the best niosomal preparation and in situ gel preparation, whereas flonida 5% was a marketed preparation available as a cream. After seeing the result based on in situ drug release, shown in (fig. 9) (table 8), proved that formulation F1 and NIG-3 are able to show a sustained release, but sustained release pattern followed by NIG-3 formulation is more appropriate and can be able to wide stand for more than 12 h of drug release, as only 80.891 % of drug release occur after 12 h of a time period, this means both the formulation (F1 and NIG-3) have the capability to sustain the drug release pattern and suitable to those patients which have a high probability of dose-related side effects and to reduce the dose frequency. Whereas when we look out the pattern of drug release of flonida 5% cream, the whole release the drug occurred within 3 h of a time period, out of which 69% attained within 1 hr of release which is proof that flonida follows an instantaneous drug release pattern in comparison with other two formulations and best suitable for a condition when a patient requires a small dose at different time period without facing any difficulty.

CONCLUSION

On the basis of different parameters of evaluation, 5-Fluorouracil loaded niosomal *in-situ* gel (NIG-3) is considered as an effective and better substitute against parenteral and oral dosage form required in the treatment of mouth cancer. After seeing the comparative *in vitro* drug release study in between all three formulations (Flonida 5%, F1 and NIG-3), one can conclude that all three formulations are capable to treat oral cancer, but flonida gel shows an instant action whereas F1 shows high permeability and sustained effect but unable to adhere over the buccal area because of which, higher amount of drug release did not achieve through the desired route, that's the reason NIG-3 selected as best among the rest because of its higher permeability, better-sustained release and higher residential time. Formulation NIG-3 is a combination of niosomes and *in-situ* gel which together enhance the sustained release effect of formulation and help to reduce the dosing frequency. There are a few additional benefits like, non-irritability, fast sol to gel conversion, apply to the desired area (all over the mouth), ease to use which lead to overall better patient compliance.

LIST OF ABBREVIATION

5-FU = 5-Fluorouracil, P-407 = Poloxamer 407, P-188 = Poloxamer 188, C-934 = Carbopol 934 P, MC = Methyl cellulose, HPMC = Hydroxypropyl methyl cellulose, F1 = Niosomal Formulation, NIG = Niosomal-*in-situ* gel.

ANIMAL RIGHTS ETHICAL APPROVAL

The Goat's tissues samples were collected from a registered slaughterhouse, and therefore the study did not require any approval from IAEC institutional animal ethical committee/CPCSEA New Delhi. But all procedures were taken under the monitoring of pharmacologists/animal handling experts.

CONSENT FOR PUBLICATION

Not applicable

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AUTHORS CONTRIBUTIONS

Both authors have contributed substantially to the experimental design, performance, analysis, and reporting of work. Dr. Anirudh Singh Deora has contributed to conceptualization, proofreading, and correspondence. Rahul Kumar Singh has conducted all related bench-work with a literature survey, draft writing, manuscript writing, and formal analysis.

CONFLICT OF INTERESTS

The authors declare no conflict of interest in article

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