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RESEARCH ARTICLE

Preparation and Characterization of Protein Loaded Microemulsion for Nasal Delivery System

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ABSTRACT

The objective of this study was to prepare and characterize a stable microemulsion system for encapsulating a Parathyroid hormone (PTH). To get the therapeutic amount PTH loaded into the microemulsion system without compromising the stability of microemulsion system, the key objective of this work was to solubilize higher percentage of aqueous phase loading with taste masking agent. (≥25%). Preformulation studies have been performed to prepare ternary phase diagram for surfactants, oils, co surfactants. Some of the critical parameters like proportion of surfactant and co-solvent, and speed of stirring were optimized to get maximum loading efficiency and *in vitro* drug release. The results of the study had conclusively demonstrated that batch F22 has given 92 % of drug release within two hour and was stable for minimum 95 days with 35.61% loading after 95 days.

KEYWORDS

Protein, Microemulsion, Parathyroid Hormone, Capmul MCM, Nasal Delivery

INTRODUCTION

Microemulsion is defined as dispersed system consisting of surfactant, co-surfactant and oil and an aqueous phase at appropriate ratios[1]. It is a thermodynamically stable and optically transparent isotropic liquid solution with a droplet diameter usually less than 100nm[2]. Unlike coarse emulsions micronized with external energy microemulsions are based on low interfacial tension. This is achieved by adding a co-surfactant, which leads to spontaneous formation of a thermodynamically stable microemulsion. Several interesting characteristics of microemulsions, namely, good thermodynamic stability, ease of preparation, high drug loading capacity, low viscosity, enhanced drug solubilization, and small droplet size, have drawn attention for their use as vehicles for drug delivery[3-5]. Microemulsions is very versatile systems and can be used to deliver drugs via several routes, but these have been extensively studied as vehicles for trans-dermal administration and have been in focus in recent years.[1,4,6-9]

As vehicle for transdermal systems, micro emulsions can increase the local or systemic delivery of drugs by different mechanisms. First, their composition and structure enable them to incorporate a greater amount of drug than other conventional topical formulations such as ointments, creams, gels, and lotions. Second, the diffusional barrier of the skin may be modified depending on the composition of the microemulsion. Third, an increased thermodynamic activity of the drug may favour its partitioning into the skin. Transnasal administration of drugs in diverse dosage forms such as sprays, powders, and microspheres has

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been attempted for improved residence and bioavailability. The nasal delivery is receiving attention for management of postoperative pain; mucosal administration requires only a 1.1-1.5 time higher dose of fentanyl than i.v. dose. Nasal drug delivery system carries various advantages like easy accessibility which facilitate self-medication, good penetration power with rapid absorption and fast onset of action, avoidance of first pass metabolism, etc.

Microemulsions are system thermodynamically stable which act as super solvent system. They can solubilize hydrophilic and lipophillic drugs including drugs that are relatively insoluble in both aqueous and hydrophobic solvents. The use of microemulsion as delivery systems can improve the efficacy of a drug, allowing the total dose to be reduced and thus minimizing side effects. Microemulsion increases the rate of absorption, increases bioavailability, and eliminates variability in absorption.

Generally, pharmaceutical micro emulsions contain additional components such as a cosurfactant and/or drug. The co-surfactant is also amphiphilic with an affinity for both the oil and aqueous phases and partitions to an appreciable extent into the surfactant interfacial monolayer present at the oil-water interface.

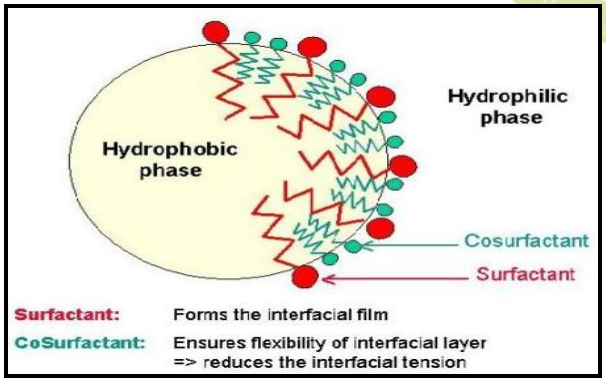

Figure 1: Schematic representation of structure of Microemulsion

A large number of drug molecules are by themselves surface active and they are expected to influence phase behavior. For four or more components, pseudo ternary phase diagrams are used to study the phase behavior. In this diagram a corner will typically represent a binary mixture of two components such as surfactant/co-surfactant, water/drug or oil/drug.

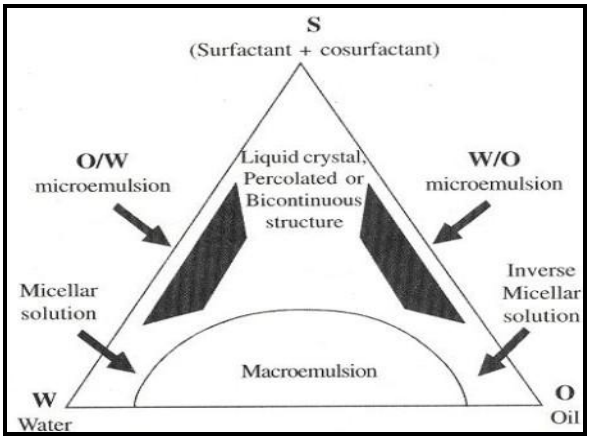

Figure 2: Hypothetical phase diagram

From the figure 2, we can see that with high oil concentration surfactant forms reverse micelles capable of solubilizing water molecules in their hydrophilic interior. Continued addition of water in this system may result in the formation of W/O microemulsion in which water exists as droplets surrounded and stabilized by interfacial layer of the surfactant / co-surfactant mixture. At a limiting water content, the isotropic clear region changes to a turbid, birefringent one. Upon further dilution with water, a liquid crystalline region may be formed in which the water is sandwiched between surfactant double layers.

Finally, as amount of water increases, this lamellar structure will break down and water will form a continuous phase containing droplets of oil stabilized by a surfactant / co-surfactant (O/W microemulsions).

MATERIALS AND METHODS

Materials

Parathyroid hormone was obtained as gift sample from INTAS Pharmaceuticals, Ahmedabad, India. Oils and Surfactants from Abitec and Croda, USA; Acrylamide, Bis Acrylamide, Ammonium per Sulfate, Glycerol, SDS, Bromophenol blue, Trizma Base, Tricine were obtained from Sigma, USA; Butanol from Merck, Germany; Glacial Acetic acid from J.T.Baker; Eriochrome Black-T from SRL; Methanol from Merck, Germany; Sodium

Thiosulphate from Merck, Germany; Sodium Hydroxide from Merck, Germany.

Methods

Required quantities of Oil, high HLB surfactant, low HLB surfactant, were accurately pipetted in to a clean glass vial. The vial was vortexed thoroughly to mix all three ingredients properly to get a clear transparent oil phase. The required amount of aqueous phase was added drop wise using micropipette in to the vial while vortexing till a clear transparent solution formed. Samples were then evaluated for stability by visual appearance and globule size measurements.

Construction of Pseudoternary Phase Diagram

Microemulsions were prepared by using conventional titration method. The oil (isopropyl myristate or oleic acid) and aqueous phases were first combined with the surfactant (Capmul and Polysorbate 80). Cosurfactant (ethanol) was added gradually with magnetic stirring at room temperature until the system was transparent. Transparent, single-phase formulations were indicative of stable microemulsions.

Microemulsions were allowed to equilibrate with gentle magnetic stirring for 15 minutes. These microemulsions were then titrated with water using at room temperature. Then, these were stirred vigorously for a sufficient length of time and end point (onset of turbidity or phase separation) was visually monitored against a dark background by illuminating the samples with a white light. The experiments were performed in triplicate to check reproducibility. From the end point composition of titrated samples, the mass percent composition of the components like oil, surfactant, and water was calculated and plotted on triangular coordinates to construct pseudoternary phase diagrams[10].

From the microemulsion regions in the pseudoternary phase diagram, the three different formulas for the development microemulsions were selected and prepared. Pseudo ternary phase diagrams were constructed to examine the formation of water in oil microemulsion using 4 components: oil, surfactant, co-surfactant, aqueous phase. The phase diagram study shows the stable microemulsions region where maximum drug loading can be obtained.

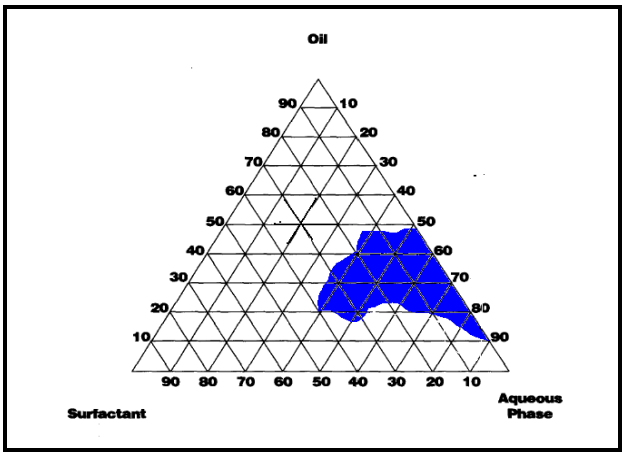

Figure 3: phase diagram of F (22) formulation

From a formulation viewpoint, the increased oil aqueous phase content in microemulsion may provide a greater opportunity for the solubilization of protein in microemulsion. Moreover, when the composition of surfactant mixture in a microemulsion preparation was <30%, the formulation was less viscous. So F (22) was optimum formulation.

Preparation of PTH-Loaded Microemulsions.

PTH loaded microemulsions were prepared by dissolving PTH in water and poured dropwise to oil (isopropyl myristate or oleic acid) and surfactant (Tween 80)/cosurfactant (ethanol) mixture with vigorous stirring at room temperature.

These PTH-loaded microemulsions were allowed to equilibrate with gentle magnetic stirring for 15 minutes. Then various formulated microemulsions were passed through Whatman filter paper

Aqueous Phase Loading in Optimization of Microemulsion

To optimize the maximum volume of the aqueous phase that could be loaded in the different microemulsion system, these microemulsion were prepared by using the High HLB surfactant, low HLB surfactant, co-

surfactant and taste masking agent with their limit.

Development of microemulsion was done by checking parameters like effect of surfactant, effect of co-surfactant, taste masking agent, different oil concentration.

Table 1: Aqueous phase loading in optimization of microemulsion

Observations		
Formulation code/Ingredients	**F(22)**	**F(22) for 18.250ml**
Oil	100µl	5ml
Low HLB surfactant	30µl	1.5ml
High HLB surfactant	50µl	2.5ml
Co-surfactant	50 mg	2.5ml
Taste masking agent	5µl	250µl
Drug loading	130µl	6.5ml
Total quantity	365µl	18250µl

Table 2: Composition of microemulsion

Formu^{n} Code	**Oil phase**	**Surfactant+ Co-surfactant**	**Aqueous phase buffer**
F1	22%	60%	18%
F2	25.57%	69.75%	4.64%
F3	26.18%	71.4%	2.38%
F4	13.63%	68.16%	18.18%
F5	21.15%	57.69%	21.15%
F6	29.08%	54.54%	16.36%
F7	35.70%	30.94%	33.33%
F8	38.95%	29.86%	31.16%
F9	37.5%	30%	32.5%
F10	33.32%	40.71%	25.92%
F11	47.36%	44.73%	7.84%
F12	26.31%	60.86%	13.15%
F13	29.41%	52.92%	17.64%
F14	35.71%	46.41%	17.85%
F15	32.25%	48.36%	19.35%
F16	23.25%	53.46%	23.25%
F17	25%	45%	30%
F18	32.25%	48.36%	19.35%
F19	30.54%	36.09%	33.33%
F20	34.37%	40.61%	25%
F21	36.05%	42.61%	21.31%
F22	28.75%	35.59%	35.61%
F23	31.94%	36.09%	33.33%
F24	32.38%	36.61%	30.98%
F25	34.76%	37.67%	27.53%
F26	33.33%	54.54%	12.12%
F27	28.56%	51.93%	19.48%

In Vitro Release Studies for Protein 'PTH' Loaded Microemulsion

In Vitro Release Study Using Dialysis Bag

1 ml each of F(22), 250 µg/ml PTH loaded microemulsion and PBS PH 7.4 was taken together in an overnight soaked dialysis bag and sealed properly. This bag was dipped in 10 ml PBS PH 7.4 as release medium and placed in

shaking water bath to maintain continuous shaking and temperature of 37ºC. 1 ml of the release medium was withdrawn and replaced with fresh PBS PH 7.4 at each time points like 0, 10, 20, 30, 40, 50, 60, 90, and 120 min. and mixed properly.

Each withdrawn samples was then analyzed by area at 492 nm in RP-HPLC to check its concentration. Area reading was used to plot calibration curve and compared with the standard curve for Protein in PBS PH 7.4 to find out the % release in a particular time.

Table 3: % In vitro release data of F(22) formulation

Sample Withdraw/min	% Cumulative Release
0	0
10	10
20	52
30	54.5
40	66.5
50	70
60	72
90	76.2
120	92

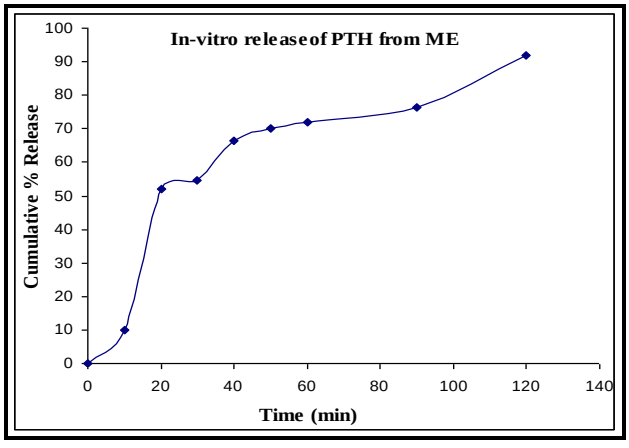

Figure 4: In vitro-release of protein from ME

From this figure 4, it is evident that about 50% of drug (protein) release from microemulsion within 20 minutes, followed by % cumulative release of drug over a period of 2 hours. It took 2 hours to release about 92% of drug from microemulsion.

Entrapment Efficiency by RP-HPLC of PTH Loaded Microemulsion

The PTH loaded (250µg) in the microemulsion was extracted and then analyzed by RP-HPLC to check its concentration, profile and retention time. The PTH had been extracted from 2 sets of microemulsion of same composition and compared with the chromatogram of the actual bulk of protein X. The mobile phase (buffers) used were TFA in WFI and TFA in acetonitrile.

$$\text{Encapsulation Efficiency (EE) \%} = \frac{\text{Total drug incorporated} - \text{Free Drug} * 100}{\text{Total Drug}}$$

Globule Size

The globule size of the microemulsion was determined by photon correlation spectroscopy which analyses the function in light scattering due to Brownian motion of particles using zetasizer, nano-25 (Malvern instrument 2000) able to measure sizes between 10 to 5000 nm. Light scattering was monitored at 25ºC.

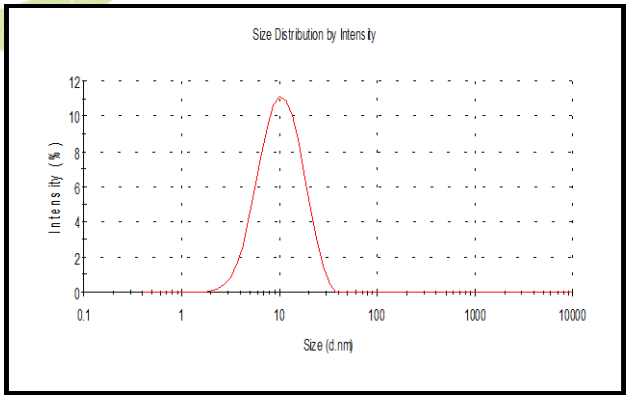

Figure 5: Globule size

RESULTS AND DISCUSSION

In the present study, three different types of oils were used for development of formulation as shown in table. From these three formulations, according to maximum aqueous phase loading capacity, we selected oil-1 for further study and evaluated for stability by visual appearance.

F(22) formulation was optimized with oil-1 having 18 % of aqueous phase loading. It clearly appears from the data that with increase in concentration of surfactant there was a simultaneous increase in aqueous phase loading. As we increased the surfactant concentration from 35µl to 50µl the aqueous phase loading get increased from 31.16% to 33.33%. So, 11.90% of surfactant was optimized for the HLB system. It clearly appears from the data that with increase in concentration of surfactant there was a simultaneous increase in aqueous phase loading. As we increased the surfactant concentration from 35µl to 50µl the aqueous phase loading get increased from 31.16% to 33.33%. So, 11.90% of surfactant was optimized. In this study, we observed the effect of ethanol as co-solvent on loading of 20µl and 50µl Different concentration of ethanol was added to achieve maximum aqueous loading. As observed with formulation that without addition of ethanol, 17.85% of aqueous phase was loaded. When we added 20µl of ethanol, aqueous phase loading get increased to 19.35%. However, there was no increase in aqueous phase loading, when we increased the ethanol volume from 20µl to 50µl. If ethanol was added

50µl, 17.64 % aqueous buffer can be loaded. From these results, 20µl of ethanol was optimized.

In the present study, it was found that when we added 20µl of isopropyl alcohol, 19.35% of aqueous phase was loaded. When we increased the isopropyl alcohol from 50µl to 100µl the concentration of aqueous phase also decrease from 30% to 23.25%. From this we concluded that with increase in the amount of isopropyl alcohol, we cannot enhance amount of aqueous phase loading and drug. On the basis of results of stability and Organoleptic properties, peppermint oil was selected.

Two formulations (F(22), F(23)) were selected with high aqueous loading and good organoleptic properties and observed that 5µl, 10µl, 15µl, 20µl respectively was optimum quantity of taste masking agent. Amount of 50µl high HLB surfactant with 5µl taste masking agent makes good combination and gives maximum loading. From the visual appearance and globule size it was clear that microemulsion system with 1 month stability microemulsions were stored at 2-8ºC and 25ºC and evaluated for stability by visual appearance and globule size for a period of one month.

Determination and of Aggregation Degradation of Protein in Microemulsion

The protein extracted from the microemulsion was analysed by SDS-PAGE to check for any aggregation or degradation, and the results is shown in the fig 5.

The lanes 1 and 5 lanes are contain IRS. The lanes 2 to 4 shows the samples extracted from microemulsion F(22) (set 1) in the order of their extraction, i.e., it shows the first, second .third and fourth fraction of extraction respectively. The lanes 6 to 8 shows the first, second .third and fourth fraction of extraction respectively for the F(22) (set 2) microemulsion. The samples from both emulsions were showing similar patterns in band and no aggregation or degradation was observed after 1 month of incubation at 2-8°C.

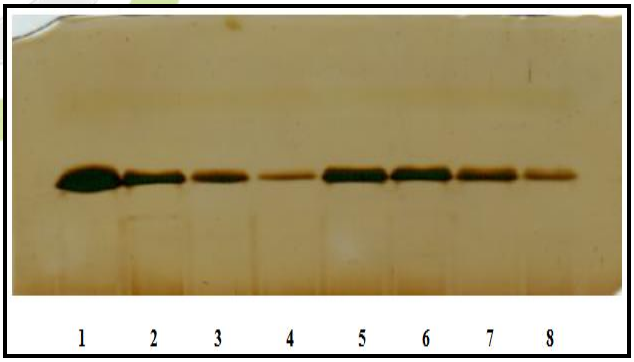

Figure 6: Aggregation and degradation of protein in microemulsion

The lanes 1 and 5 lanes are contain IRS. The lanes 2 to 4 shows the samples extracted from microemulsion F(22) (set 1) in the order of their extraction, i.e., it shows the first, second .third and fourth fraction of extraction respectively. The lanes 6 to 8 shows the first, second .third and fourth fraction of extraction respectively for the F(22) (set 2) microemulsion. The samples from both emulsions were showing similar

patterns in band and no aggregation or degradation was observed after 1 month of incubation at 2-8°C.

CONCLUSION

From this study and research on microemulsion, the following datas are optimized and can be concluded as:

Oil-1 was selected for further study, which having maximum aqueous phase loading and was stable for one month. As we increased the surfactant concentration from 35µl to 50µl the aqueous phase loading get increased from 31.16% to 33.33%. 11.90% of surfactant was optimized for the HLB system. So, we conclude volume of 50µl was optimized quantity for surfactant and 50mg was optimized quantity for co-surfactant.

From effect of co-solvent, we can concluded that optimum concentration of ethanol is 20µl and as we increased the amount of isopropyl alcohol, we cannot enhance amount of aqueous phase loading but no major role of iso propyl alcohol in microemulsion.

In the microemulsion system, combination of low HLB surfactant with high HLB surfactant it gives good taste. Two formulations (F(22), F(23)) were selected with aqueous loading and good organoleptic properties and we concluded that 5µl respectively optimum quantity of taste masking agent. Vortexing was optimized as the method of mixing for preparation of microemulsions. Almost up to 35.61% of the aqueous phase could be loaded into the F(22) formulation with additional surfactant maintaining the stability for minimum of 30 days at 2-8 ºC. The microemulsion was stable till 35.61% of aqueous phase with 250µg/ml protein loading.

F(22) formulation was stable for minimum 95 days with 35.61% loading.

The microemulsion doesn't show much pH fluctuation recommending its stability. The in vitro release study shows 92 % of release of protein into PBS buffer within two hour. From RP-HPLC data it is clear that the retention time for the actual bulk of protein 'X' and that of the extracted protein from two sets of microemulsion was almost same suggesting that the protein in the microemulsion is stable.

From the SDS-PAGE results it is clear that the protein had not undergone any aggregation or degradation inside the microemulsion.

Firstly, the formulation F(22) prepared for 1.6 ml with protein loading 250µg/ml. And bulk concentration was 0.9 mg/ml. microemulsion was prepared by general method preparation as described earlier.

The sample was stored at 2-8°C and evaluated for stability by visual appearance and globule size. The protein loaded (250µg) in the microemulsion was extracted and then analyzed by RP-HPLC. Check its % purity form recovery, globule size and visual appearance. It was measured at different time points like initial day, 4^{th} day, and 11^{th} day, 32 day.

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