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Preparation and *in-vitro* evaluation of Carbopol hydrogel of clobetasol-loaded ethylcellulose microsponges

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ABSTRACT: Clobetasol propionate (CP) is a potent corticosteroid used for skin conditions but often causes side effects due its systemic absorption. To improve its solubility and reduce it side effects (like skin irritation, skin atrophy, hypopigmentation and steroidal acne), Microsponge (Msg) has been employed as a unique three-dimensional particle that can encapsulate hydrophilic and lipophilic drugs. This study aims to develop and evaluate CP Msg-loaded hydrogels. Two Clobetasol-loaded ethylcellulose-based Msg formulas were prepared using the quasi-emulsion solvent diffusion method, then they were incorporated into Carbopol hydrogel. Two ratios of Carbopol 940 (1% and 1.5% w/w) were used. The prepared hydrogel were assessed for appearance, pH, drug content, spreadability, extrudability, rheology, and in vitro release. The optimum hydrogel was compared to generic CP cream available locally and plain hydrogel. The results showed that both Msg formulas had good product yield, entrapment efficiency and highly porous micron size. The four prepared hydrogels revealed acceptable characterization including; pH ranged between 5.6and 6, drug content (98.8- 100%) and % extrudability (80.7-92%) with pseudoplastic flow type. The hydrogel formula (F2Ha 1%) containing (1:1 weight ratio of CP: ethylcellulose) with (1%w\w Carbopol) was chosen as the optimized formula since it showed the highest spreadablility and approximately 43% of CP was released at 8 hours. The ex-vivo data including; the highest deposition in stratum corneum and epidermal/ dermis with the flux, permeability coefficient and lag time of F2Ha were low, compared to plain hydrogel and marketed cream. Based on the study's finding, we concluded that CP Msg-loaded Carbopol hydrogel is a proper drug delivery system for topical application with minimized systemic absorption.

KEYWORDS: Carbopol; clobetasol propionate; ethylcellulose; hydrogel; microsponges.

1. INTRODUCTION

Microsponges (Msg) are drug delivery systems made up of small, porous microspheres. These microspheres resemble tiny sponge-like particles with a substantial porous surface [1]. Msg provides many benefits over other delivery systems, such as control drug release and improvement of physicochemical and thermal drug stability[2].

Ethylcellulose (EC) is used to prepare microsponge because of its stability, flexibility, and low cost. Microsponge prepared with EC indicated high chemical stability in the study of Bothiraja C et al. on eberconazole nitrate, and Sheikh A et al. on celecoxib. In both studies, the scan electron microscope images showed the creation of microsponge which appear with many pores on surfaces, also it shows uniform microsponges particle size and shape. The studies have concluded that successfully utilized of ethylcellulose for the formulation of microsponges using the emulsification solvent diffusion method [3,4].

Microsponges are beneficial to cover drug side effects and provide sustained drug release; however, Msg can't be applied topically until it's incorporated in the hydrogel, Hydrogels are water-swollen three-dimensional networks of polymers, proteins, small molecules or colloids. They constitute a versatile platform for drug delivery because of their capacity to encapsulate and protect drugs and provide sustained and remotely programmable spatial and temporal release [5]. In the study of oxybenzone microsponge hydrogel that prepared by Pawar A P and his research group, the *in-vitro* and *ex-vivo* evaluation study of microsponge hydrogel revealed remarkable enhacement of topical drug retention for prolonged period of time [6].

Clobetasol propionate (CP) is widely used to treat various skin disorders, including vitiligo, atopic dermatitis, pruritic eczema, and psoriasis. However, it has many side effects when applied to the skin, like

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skin atrophy, hypopigmentation, steroidal acne, and allergic contact dermatitis [7]. CP belongs to class II in biopharmaceutical classification system (BCS), which means it has low solubility and high permeability. CP is white to creamy-white crystalline powder. Its melting point is 195.5-197 °C; practically insoluble in water; soluble 1 in 100 of ethanol and 1 in 1000 of ether. Log P (octanol/water) is 3.5 [8]. Devi and her research group formulated Eudragit® RS 100 microsponges to minimize challenges associated with CP and to improve its delivery characteristics. The microsponges were formulated via quasi-emulsion solvent diffusion technique using Eudragit® RS 100, polyvinyl alcohol, and dichloromethane[9]. Nausheen and her research group develop a bigel-containing clobetasol propionate-loaded PLGA microparticles developed by solvent evaporation method. The study findings suggested that clobetasol microparticle-loaded bigel formulation appeared to be a promising therapy option for psoriasis[10].

The aim of this study is to incorporate CP-loaded ethylcellulose-based microsponges in Carbopol hydrogel using two concentrations (1% and 1.5% w/w) for topical application. All tests for evaluating the quality of prepared hydrogels were done, which included pH determination, spreadability, extrudibility, viscosity measurement and CP in-vitro and ex-vivo released study.

2. RESULTS

2.1. Preparation of CP microsponges

Dichloromethane was used as an internal phase organic solvent because it is a good solvent for both the drug and the polymer, in addition to its easy evaporation after diffusion, leaving a solid CP microsponge. Quasi-emulsion solvent diffusion method was used since it is easy to perform [11].

2.2. Characterization of CP microsponge-prepared formulations

Msg properties were displayed in Table 1, including production yield, % drug entrapment, and particle diameter.

Table 1. Characterization of Microsponges Formulas

Formula code	Product yield %	Drug Entrapment %	Particle diameter (µm)
F1	77.77±0.6	77.41 ±2.04	55.97± 0.9
F2	69.00±0.8	64.7±1.56	62.51±1.5

As shown in Table 1, CP entrapment % had the same behaviour as product yield; when increasing the polymer: drug ratio led to a significant decrease ($p< 0.05$) in production yield from 77.77% to 69% and CP entrapment % from 77.41 ±2.04 to 64.7±1.56 % for formula F1 and F2, respectively. This may be explained by the fact that decreasing the amount of polymer concurrently increases the drug amount used, leading to a decrease in the viscosity of the medium, which allows easier diffusion of drug moiety and the formation of a more flexible polymer coat and more drug entrapped [12,13]

On the other hand, when the polymer: drug ratio increases, the particle size will increase; this is because when increasing polymer: drug ratio, more amount of the polymer will be available to form CP-Msg, which will increase the thickness of the polymer will lead to the formation of large size Msg [14]

2.2.1 SEM

SEM analysis of pure drug illustrated the crystal structure of CP, which was confirmed by DSC thermogram. The SEM analysis of formula F1 and F2 showed spherical porous particles with uniform size and shape; the surface has many pores, as this is the effect of solvent diffusion, as shown in Figure 1. This study is identical to the result of Fareed NY and Kassab HJ on acyclovir microsponges [15]

2.2.2 DSC

DSC thermogram of pure CP showed that CP having a sharp endothermic peak at 198°C corresponds to the melting point of CP in crystal form [8]. EC polymer has glass transition temperature (Tg at 128.7°C) indicate amorphous nature and increase in fluidity after Tg. At higher temperature another peak (223.3°C) was appeared in EC thermogram due to melting of microcrystal particles. The presence of CP peak in the physical mixture indicated good compatibility between CP and the selected polymer (EC). For the formula (F1 and F2), the DSC thermo gram revealed the disappearance of the CP peak which may due to decrease its crystallinity and converted to amorphous form that uniformly dispersed in EC matrix. The EC peaks are present in formulas (F1 and F2) thermogram, as shown in Figure 2.

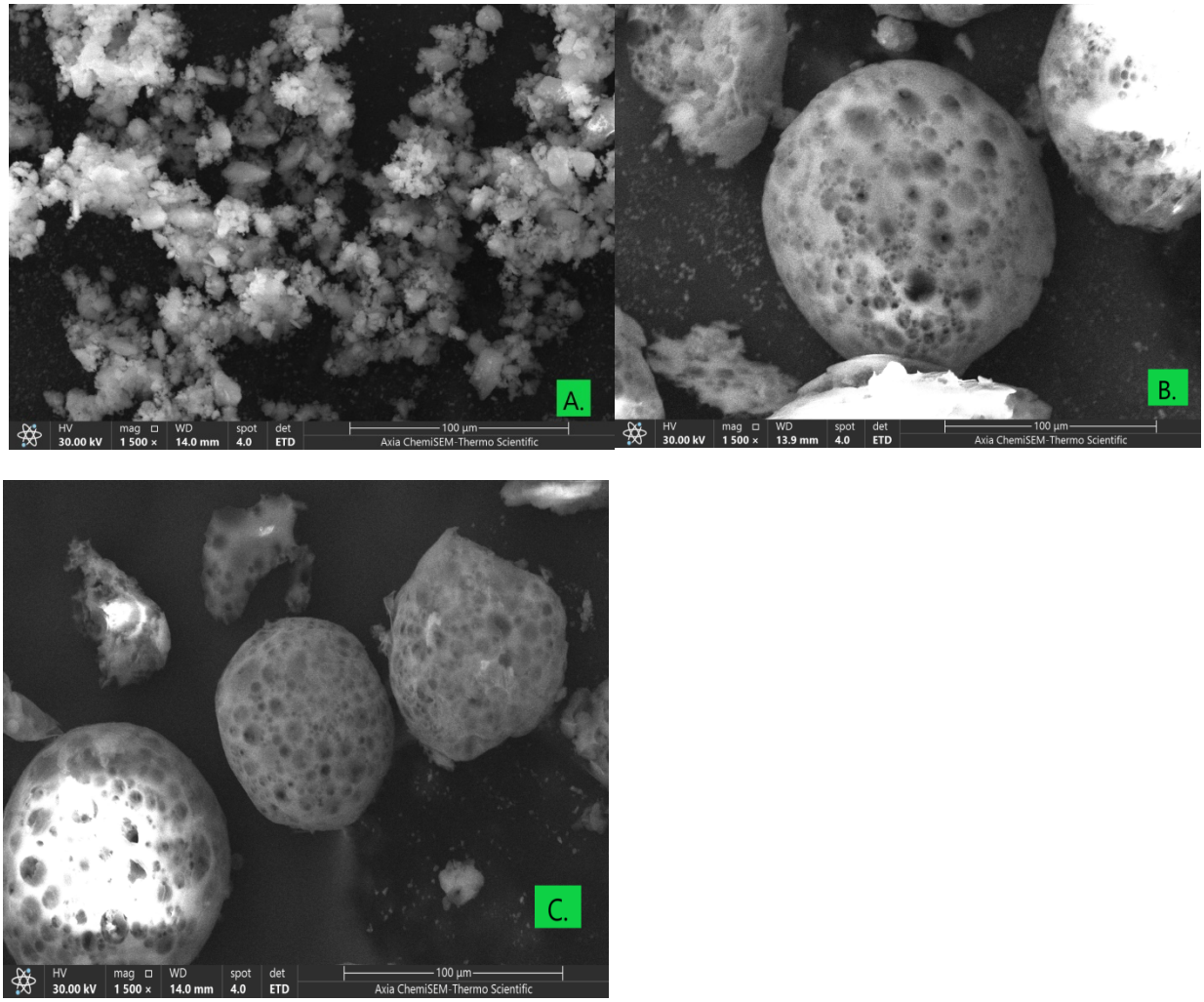

Figure 1. SEM pictures at 1500 mag of A) CP. B) F1 and C) F2.

2.3. Evaluation of the prepared hydrogel

2.3.1. The visual examination

Visual inspection of the physical appearance of all the prepared CP-Msg hydrogel revealed that the formulas had good homogeneity, free of any grittiness, and there was no phase separation. Formulas appeared transparent with no obvious color.

2.3.2. pH Determination

The pH values of prepared CP Msg hydrogel formulas are shown in Table 2. The results ranged between 5.6±0.23 and 6±0.5, which are acceptable for applying on skin without irritation.

2.3.3. Determination of Drug Content

CP content in four of the prepared CP-Msg hydrogel were reported in Table 2. The practically determined drug content ranged from (98.8±0.32 to 100±0.65%) of the theoretical content**.** This indicated the uniform dispersion of CP-Msg through the prepared hydrogel structure.

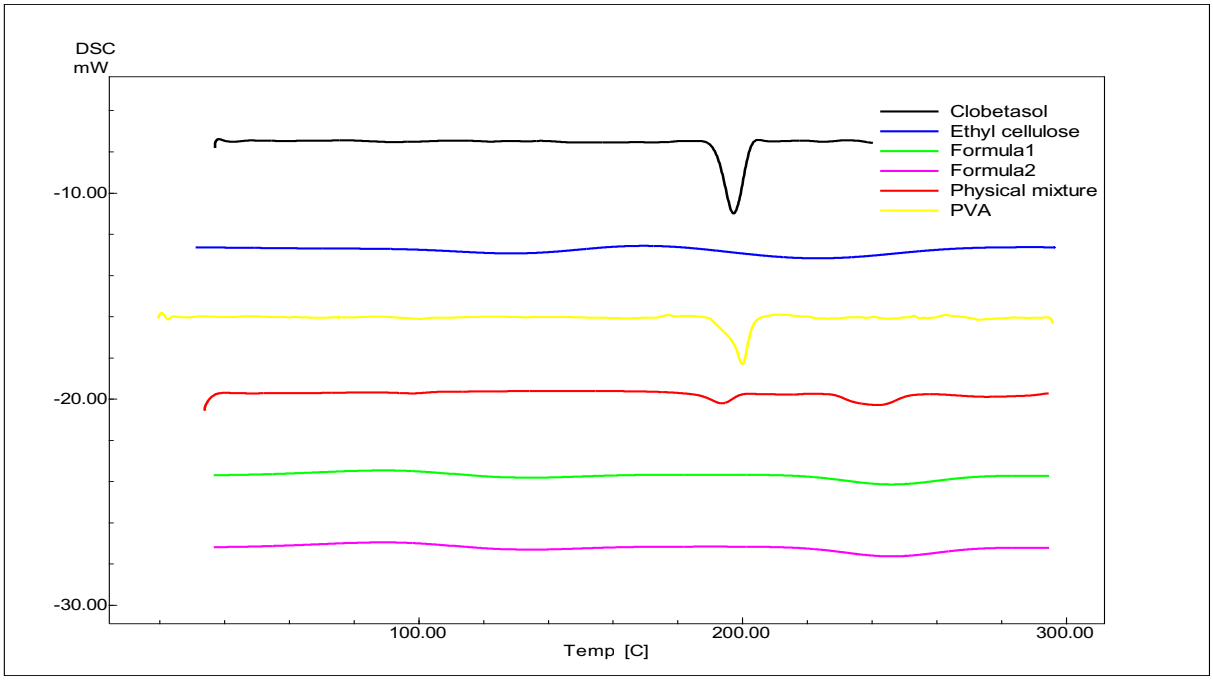

Figure 2. Thermogram obtained by DSC.

2.3.4. Spreadability Determination

Spreadability is an important property of topical formulation from a patient compliance point of view. Application of the formulation to the skin is more comfortable if the base spreads easily. The large diameter indicates better spreadability [16]. Spreadability values of the prepared CP-MSg-based gel were reported in table 2.

2.3.5. Extrudability Determination

The extrudability values were considered as good for all prepared hydrogel formulas since all obtained values are above 80%, as shown in Table 2.

Table 2. Measured PH Values, Drug content, Spreadability and Extrudability of CP- Msg Loaded Hydrogels

Formula code (Carbopol 940® w/w %)	PH	%Drug content	Spreadable Diameter (cm)	% Extrudability
F1Ha (1%)	6±0.5	99.2±0.12	7	92%
F1Hb (1.5%)	5.6±0.23	98.8±0.32	6.7	84%
F2Ha (1%)	5.8±0.31	100±0.65	7.5	90%
F2Hb (1.5%)	5.7±0.2	99.4±0.18	5.4	80.7%

2.3.6. In vitro CP Release Study from Msg Hydrogels

As is shown in Figure 3, plain hydrogel had the lowest CP releasing rate compared to CP-Msg loaded Carbopol 940 hydrogels. For each Msg formula, an increase in Carbopol concentration results in a decrease in release rate because the hydrogel is tightly cross-linked. Thus, it may not be possible for CP to penetrate the dissolution medium in significant quantities. As a result, the drug remains in the interstitial spaces where it is released slowly [17]. Comparing Msg ratio, the formulas that contain Msg ratio 1:1 (formulas F2Ha and F2Hb) showed higher release that hydrogels containing Msg ratio 2:1 (Formulas F1Ha and F1Hb).

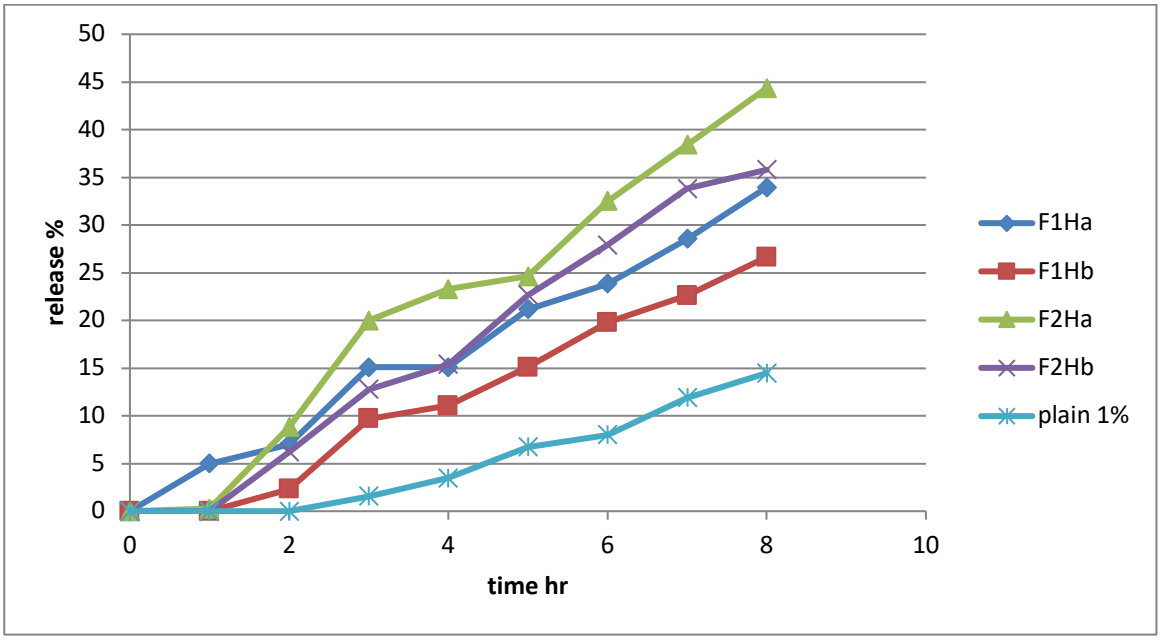

Figure 3. Release profile of CP-Msg hydrogels compared to plain hydrogel.

2.3.7. In vitro release kinetics of CP hydrogel formulas:

The release of CP from hydrogel of all formulas, include plain hydrogel, is best fit with higher correlation (R2) with zero order equation, which shows a zero-order release profile. According to kosmeyer-peppas (n value), it indicates that the drug release mechanism was non-Fickian, and suppose that CP hydrogel delivered the active ingredients by non-Fickian diffusion (see Table 3).

Table 3. In vitro release kinetics of CP hydrogel formulas:

Hydrogel code	zero order R^2	first-order R^2	Higuchi R^2	Kosmeyer-Peppas R^2	Kosmeyer-Peppas (n)
F1Ha (1%)	**0.9866**	0.9816	0.8634	0.9867	0.991
F1Hb (1.5%)	**0.9591**	0.9449	0.7571	0.9819	1.295
F2Ha (1%)	**0.9730**	0.9606	0.8230	0.9744	1.064
F2Hb (1.5%)	**0.9722**	0.9542	0.7843	0.9852	1.212
Plain hydrogel	0.8607	0.8482	0.6107	0.9711	1.834

2.3.8. Determination of the viscosity

After measuring the viscosity at different shear rates, the data are represented in Figure 4, from which it can be seen that the viscosity of hydrogel of F1Ha was ranged (179100-12500 cp), while for the F1Hb hydrogel was (221600-20900 cp). For formula F2Ha, it was (206500-15200 cp) and formula F2Hb was 524500-36900 cp. Thus, as the shear rate increased, the viscosity was decreased. Therefore, it is non-Newtonian flow behavior. The results showed that Carbopol 940 was a good gelling agent for the preparation of hydrogels [18]. Also, the increase in polymer concentration caused an increase in the viscosity of the formed hydrogel [19].

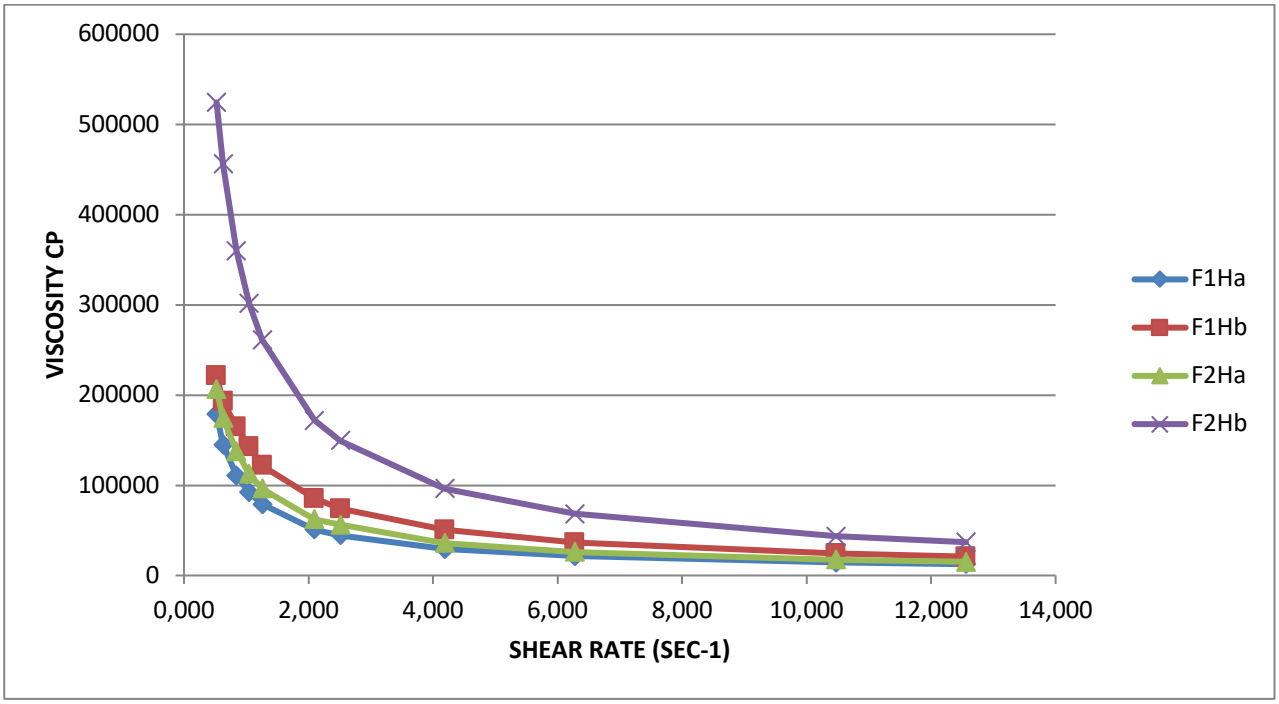

Figure 4. Viscosity versus shear rate for prepared hydrogel

2.3.9. Ex vivo CP skin permeation study

F2Ha 1% hydrogel was chosen as the optimized formula since it revealed a suitable pH value with the highest spreadability and in-vitro CP release. So, it was subjected to ex-vivo full-thickness skin permeation to be compared with locally marketed products and a plain hydrogel. Study skin permeation parameters and profiles at various time intervals are presented in Table 4 and Figure 5. The lower flux value for F2Ha compared with plain hydrogel results from Msg formation; the micron size of the Msg causes a significant decrease in the CP particle size, causing a lower flux value.

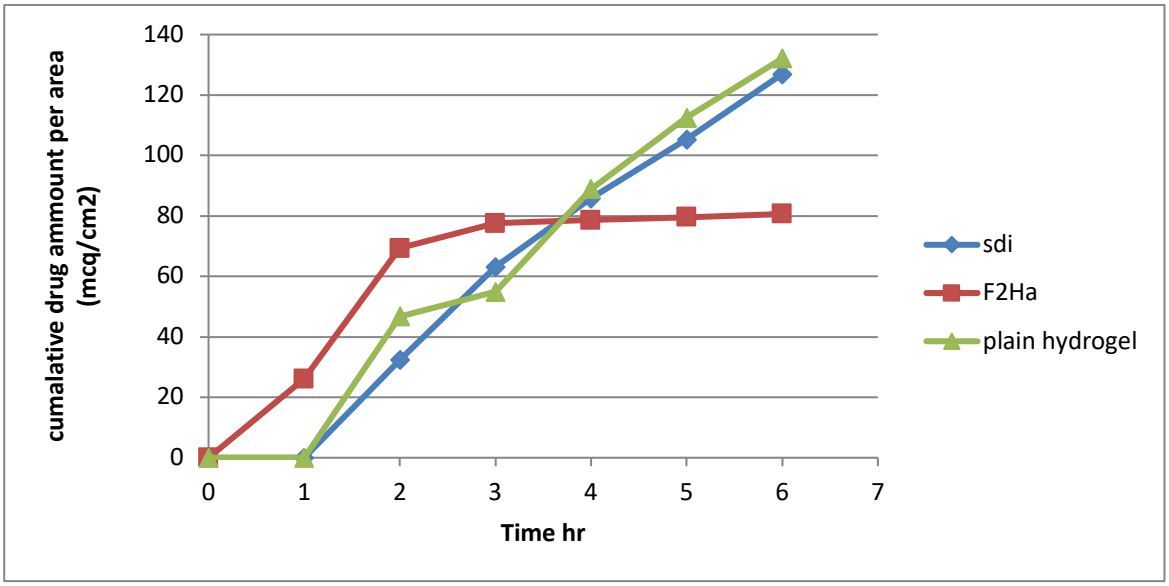

Figure 5. CP permeation profile from different hydrogel formulations

Table 4. The permeation parameters of the selected CP-Mgs hydrogel, plain hydrogel and marketed product.

Formula code	Flux (dQ/dt.S) (µg/h.cm2)	Permeability coefficient (P_{app}*10-4-cm/h)	Lag Time (hr)
DERMODIN® sdi	25.04	34	1.00
F2Ha hydrogel	2.47	3.35	1.80
Plain hydrogel	25.51	34.6	1.50

2.3.10. Stratum corneum (SC) retention and epidermis/dermis deposition study

Based on the data presented in Figure 6, F2Ha scored the highest total amount of CP in the stratum corneum after 6 h with a value of 233.7µg/cm². The same formula showed the lowest flux value, releasing 2.47*10-4 of cumulative amount of CP after 6 h in Table 4.

F2Ha formula scored the total amount of CP in the epidermis/dermis after 6 h with a value of 156.35µg/cm². As shown in Figure 6.

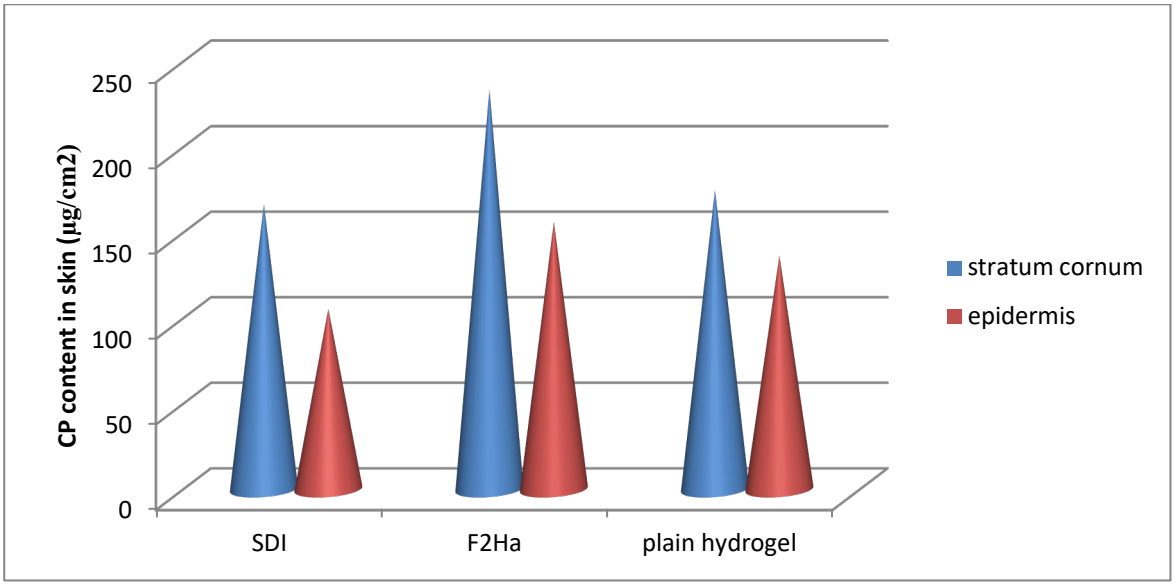

Figure 6. CP deposition into the skin

2.3.11. Skin irritation test:

A perfect drug delivery system would be able to distribute the drug without inducing toxicity, immunogenicity, or irritability. Skin irritation has been noted as one of the typical side effects following the use of CP. Additionally, it has been demonstrated that skin irritability and the concentration of the active ingredient may be directly related. According to Draize, Woodward, and Calvery, the substances that resulted in scores of 2 or lower have been viewed negatively (without any skin irritation). According to this, as represented by Table 5, the CP Msg Hydrogel did not cause skin irritations and is safe to be applied on the skin for the intended period [20]. as shown in Figure 7

Table 5. Skin Irritation Scores Following 24hr of Gel Application.

Rat no.	Controlled		Blank hydrogel		CP Msg hydrogel	
	erythema	oedema	erythema	oedema	erythema	oedema
1	0	0	1	0	0	0
2	0	0	1	0	1	0
3	0	0	1	0	0	0
4	0	0	0	0	0	0
5	0	0	0	0	0	0
average			0.6		0.2	

Erythema scale: 0, none; 1, slight; 2, well defined; 3, moderate; and 4, scar formation.
odema scale: 0, none; 1, slight; 2, well defined; 3, moderate; and 4, severe

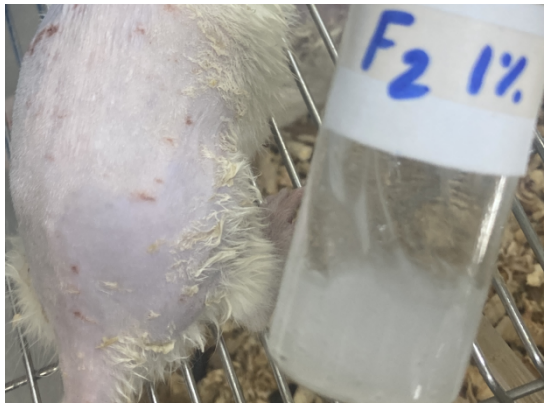

Figure 7. One of the CP Msg groups after 24 hr.

3. CONCLUSION

The results of this study suggest that incorporating Carbopol 940 at a 1% concentration leads to the development of an optimized hydrogel with favorable characteristics such as appearance, pH, spreading ability, extrudibility and the potential to reduce skin irritation while enhancing drug deposition. Importantly, the inclusion of Carbopol does not negatively impact the release of the active ingredient, CP, from the Msg. Additionally; it is observed that Carbopol is compatible with CP-loaded ethylcellouse Msg.

4. MATERIALS AND METHODS

4.1. Material

Clobetasol-17 propionate (CP) and ethylcellulose (EC) were kindly gifted by Samara Drug Industry, Samara, Iraq. Polyvinyl alcohol (PVA) was purchased from Indian Fine Chemical, India. Carbopol 940 and triethanolamine were both purchased from Himedia, India. Methanol was purchased from Loba Chem, India; it is of HPLC grade. Dialysis bag (MWco 8000-14000 D) was purchased from Special Product Laboratory, USA. Polyethylene glycol 400, Potassium dihydrogen phosphate, Disodium hydrogen phosphate, and sodium chloride were purchased from Loba Chem, India, and a Micro porous membrane of 0.22mcm was purchased from ANOW, China.

4.2. Method

4.2.1. Preparation of CP microsponges

Two formulas of CP microsponges (F1 and F2) were prepared as listed in Table 6. The quasi-emulsion solvent diffusion method was employed for the preparation. In this process, the internal phase was created by dissolving CP and ethylcellulose in 5 ml of dichloromethane through sonication using an ultrasonic shaker (Copley Scientific, UK). Simultaneously, the external phase was composed of PVA (polyvinyl alcohol) dissolved in 50 ml of distilled water. The internal phase was introduced drop by drop into the external phase. with stirring for 2 hours using hot plate magnetic stirrer (Joan lab; China) at stirring rate (500rpm) at which the organic solvent could evaporate, and solid microsponge will precipitate; the resulted MS will be collected using Buchner funnel device with a vacuum pump (Kennedy manufacturing; USA) and was washed three times with distilled water then left to dry at 40 ^{o}C in the oven (Memmert; Germany) for 12 hours [21].

Table 6. Composition of Different CP-loaded Ethylcellulose Microsponges

Formula code	CP: ethyl cellulose ratio	Aqueous (external) phase volume (ml)	Stirring rate (rpm)	PVA concentration (w/v %)
F1	2:1	50	500	0.25
F2	1:1	50	500	0.25

4.2.2. Characterization of CP microsponge-prepared formulations

The prepared two formulas were evaluated for % product yield, particle diameter; % drug entrapment; particles surface morphology(SEM) and solid state characterization (DSC) [22,23]

4.2.3. Preparing of CP hydrogel

Carbopol®940 was used as gelling agent at concentrations of 1% and 1.5%w/w to prepare Msg-based hydrogel, as shown in Table 7. Firstly, the calculated amount of Carbopol was dispersed in water using a magnetic stirrer with a speed of (500 rpm) for (30min) until uniform dispersion; the aqueous dispersion of Carbopol was allowed to stand overnight to give a chance for polymer swelling. The weight of CP ethylcellulose-microsponge equal to 25mg of the pure drug is added to polyethylene glycol (PEG 400), which is used as a humectant and a viscosity modifier. The mixture was added to 50ml distilled water that contained carbopol®940. After that, sonication for (15 min) using a water bath sonicator to allow air bubbles to escape, then a few drops of triethanolamine were added to initiate the hydrogel formation. The prepared hydrogel formulas were packed in a tightly closed container and kept at room temperature in a dark place for further evaluation tests [24,25].

Table 7. Composition of CP Microsponges Cabopol Hydroel

Formula code / Ingredients	F1Ha	F1Hb	F2Ha	F2Hb
CP microsponge equal to 25mg clobetasol (mg)	48.71	48.71	77.27	77.27
Carbopol 940 (g)	0.5	0.75	0.5	0.75
PEG 400 (mL)	1	1	1	1
triethanolamine	Few drops	Few drops	Few drops	Few drops
Distilled water q.s (g)	50	50	50	50

4.2.4. Evaluation of the prepared hydrogel

The visual examination

The examination considered a series of visual characteristics, such as consistency, color, and homogeneity [26].

Measurement of pH

The pH of the hydrogel formulations was assessed at room temperature using a pH meter. This involved immersing the glass electrode entirely into the hydrogel system and recording the pH reading [27].

Determination of Drug Content

Accurately weighted (1g) of CP-Msg loaded hydrogels that are equivalent to 0.5 mg of CP were transferred to a 50 ml volumetric flask containing methanol. The mixture was sonicated for 5 minutes. The drug content was determined by measuring the absorbance at 240 nm using a UV-visible spectrophotometer [28].

Determination of Spreadability

A weighed quantity (2 g) of CP-Msg loaded gel was placed within a circle of 3 cm diameter pre-marked on a glass plate (15 x 15 cm). Another glass plate (15 x 15 cm) was placed on the hydrogel. A weight equivalent to 500 g was placed on the upper glass plate for 5 min. It was noted that the diameter was increased due to hydrogel spreading, and then a new diameter was measured [29,30].

Determination of Extrudability

CP-Msg hydrogel equivalent to 7.5 g was filled in a feeding syringe and sealed at the end. A weight of 500 mg was applied for 30 seconds. The amount of the extruded hydrogel was collected and weighed. The per cent of the extruded hydrogel was calculated.

In-vitro Dissolution Test of CP Msg Hydroels:

To conduct the in vitro release of CP from the hydrogel formulations, a modified version of the USP dissolution apparatus (type II) was employed. A specific amount of hydrogel (3 g, containing 1.5 mg CP) was evenly applied to the surface of a dialysis membrane that had been soaked overnight in a solution of 20% methanol and phosphate buffer (pH 5.5). The membrane was fixed on the circular open end of a tube using a rubber band. The tubes were inverted and fixed on the lower part of the paddle using rubber bands in such a way that the lower end of the tube containing the gel was just immersed below the surface of a 150 ml dissolution medium as a receptor compartment that was maintained at 37 ± 2 °C for 8 hours. The paddles were set in motion at a speed of 50 revolutions per minute (rpm). At specific time intervals (30, 60, 120, 180, 240, 300, 360, 420, and 480 minutes), 5 ml samples were withdrawn and replaced with an equal volume of fresh dissolution medium to maintain a consistent volume. These samples were then filtered through a 0.45 µm filter from Millipore and subsequently analyzed for CP content using a double-beam UV-visible spectrophotometer at its λmax [31,32]

Determination of Viscosity

The viscosity of the prepared hydrogel formulations is a crucial parameter to monitor, as it serves as a fundamental indicator of the consistency and ease of application of the hydrogel formulations on the skin [33]. A viscosity test was done using a viscometer Myr VR3000, Visotech, Spain) utilizing spindle R7 [34]. The rotation speed was increased from 0 to 200; the test was done at room temperature [35,36].

Ex-vivo permeation test

A permeation study on the optimized hydrogel formula, plain CP hydrogel, and a marketed CP cream was conducted using five male Sprague–Dawley rats, weighing approximately 150–250 g each. The procedure for this experiment was approved by the Search Ethics, ensuring humane treatment of all animals involved. The process commenced by administering a euthanasia overdose to the rats and subsequently removing abdominal hair with an electric shaver. Following hair removal, careful examination was carried out to detect any bites, scratches, or irregularities on the full-thickness rat skin. Subsequently, the subcutaneous fat was gently removed without causing harm to the epidermis. The prepared skin was then soaked in a phosphate buffer saline (PBS) solution overnight before the permeation study was initiated [37]. Onto the receptor compartment, the dissolution medium is 150 of 20%methanol+PBS 7.4; the full-thickness skin was mounted with the stratum corneum side opposite to the donor chamber and then 3g of each hydrogel formula were applied into each cell. The sample was taken from the receptor compartment at time intervals 1, 2, 3, 4, 5 and 6 h. The withdrawn samples were measured at CP λ_{max}, 242 nm, using a double-beam UV-visible spectrophotometer. In this study, the cross-sectional area of the intestinal sac (S) was equal to 4.91 cm^2, and the apparent permeability coefficients (Papp) were calculated using (equations 1):

$$Papp = (dQ/dt) / (S * Co) \quad \text{..... (equation 1)}$$

Where (dQ/dt)/S is the drug flux into the acceptor solution. The attainment of a steady-state rate often referred to as flux, can be accomplished by plotting the cumulative quantity of drug that permeates across the skin membrane against time. Employing linear regression analysis on the data allows us to determine the slope of the linear segment of the graph, which signifies the flux. Meanwhile, Co signifies the initial drug concentration on the stratum corneum side [38].

Stratum corneum (SC) retention and skin deposition study

Following the permeation study, each cell's skin was thoroughly rinsed to eliminate any surplus formulation and then dried using clean filter paper. The skin samples were firmly affixed to a clean, flat surface, and the stratum corneum (SC) layer was carefully removed using 20 adhesive tape strips. To extract CP, the tape strips were individually placed in containers, each containing a group of five, and immersed in 5 mL of methanol overnight before undergoing analysis via UV spectrophotometry. These experiments were conducted in triplicate.[39]

Following the skin permeation test and the stratum corneum retention study, the rat skin underwent a 10-second rinse in distilled water to eliminate any adhering drug. Subsequently, the skin was diced into small pieces and subjected to 15 minutes of sonication in 5 mL of methanol using a bath sonicator to extract the deposited drug. The resulting samples were then centrifuged, and the clear liquid above the sediment was filtered through a 0.45 mm membrane filter before undergoing analysis by UV [40].

Skin irritation test:

The skin irritation potential of CP Msg hydrogel was investigated using the Draize patch test on rats. The experiments followed the Institutional Animal Ethics Committee's guidelines. In order to run the experiment, each rat had its dorsal area shaved prior to the experiment. Three groups of animals (n = 5) were created: Group I received the CP Msg hydrogel, group II received the plain CP hydrogel, and Group III served as the control group by precisely weighing 1 g of the hydrogels and evenly applying it over a 4 cm^2 area on the hairless skin of rats. Findings were recording after both 1h and 24 h following application of the formulations; based on the visual observation of any obvious change, such as erythema (redness) or edema, the findings of the skin irritation test were made. The sensitivity was scored [41,42].

4.3. Statistical Analysis:

The results of the experiments were given as a mean of triplicate samples ± standard deviation and were analyzed according to the one-way ANOVA at the level of significance ($P < 0.05$).

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Conflict of interest statement: The authors declared no conflict of interest in the manuscript.

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