Non-ionic surfactant based vesicular drug delivery system for topical delivery of caffeine for treatment of cellulite: design, formulation, characterization, histological anti-cellulite activity, and pharmacokinetic evaluation

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**ABSTRACT**

Cellulite is a common topographical alteration where skin acquires an orange peel or mattress appearance with alterations in adipose tissue and microcirculation. This work aims to develop and evaluate a topical niosomal gel formulae with good permeation to reach the subcutaneous fat layer. Several caffeine niosomal dispersions were prepared and incorporated into gel formulae using Carbopol 940 polymer, chemical penetration enhancers, and iontophoresis, then the prepared gels were applied onto the skin of rats and ant cellulite activity of caffeine from the prepared gels compared to that of the commercial product Cellu Destock. The results of histology revealed reduction in size and thickness of fatty layer of rat skin in the following order: FVII > FXIV > Cellu Destock > FVI + Iontophoresis > FXIV + Iontophoresis. Pharmacokinetic results of caffeine in plasma revealed that \( C_{\text{max}} \), \( T_{\text{max}} \), and \( \text{AUC}_{0-12h} \) decreased in the following order: FXIV > FVI + Cellu Destock. These results conclude that incorporation of caffeine niosomal dispersion into gel matrix with penetration enhancers and iontophoresis resulted in improvement in penetration of caffeine through the skin into the underlying fatty layer in treatment of cellulite.

**Introduction**

Cellulite is considered a non-inflammatory, degenerative phenomenon that provokes alterations to the hypodermis which causes abnormal changes in the layers of skin beneath the affected areas [1]. Evidence of the hormonal influence on cellulite is its presence in most women, its usual onset at puberty, and its exacerbation during pregnancy, nursing, menopause, and its connection with oral contraceptive use [2,3]. Topical anti-cellulite treatments include enhancement of microcirculation, reduction of lipogenesis, revival of normal skin structure, and scavenging of free radicals [4]. Many drugs were used for the management of cellulite, of which aminophylline and retinoids were evaluated [2]. Methylxanthines, such as Caffeine, aminophylline, theophylline, and theobromine, as \( \beta \)-agonists and are the main category with \( \beta \)-adrenoceptor agonist effect [12]. Caffeine stimulates lipolysis by increasing the cAMP levels in adipocytes. Caffeine takes part in the reduction of cellulite [6]. Caffeine has high water solubility of 20 mg/ml and log \( P \) of \(-0.07\) which indicate its poor skin permeation [7], that is why it is a hydrophilic model drug in transdermal experiments [8].

Niosomes are vesicular system formed of nonionic surfactants, made more stable by the presence of cholesterol [9]. Because of their amphiphilic nature, niosomes can entrap either hydrophilic drugs in their cavity or hydrophobic ones in the less polar region [10]. Niosomes are similar to liposomes but are more chemically stable [11–13]. Because of the potential of niosomes to carry a variety of drugs, these vesicles have been widely used in various drug delivery systems like drug targeting, controlled release, and permeation enhancement of drugs [14]. Administration of drugs in the form of niosomal vesicles prolongs their residence time in circulation and target their effect to the required tissues [15].

Gel dosage form is highly suitable for electrically assisted topical and transdermal permeation of drugs [16]. The high proportion of water employed in gel formulae can in turn provide an advantageous electro-conductive base for clinical use [17]. Combination between any two enhancement techniques is thought to have advantage in increasing drug permeation and decreasing the side effects of any of the techniques used alone. Iontophoresis in conjunction with chemical enhancers produce a synergistic effect on the permeation of many drugs [18–20].

The goal of this study is to prepare Caffeine niosomal dispersions, evaluate their entrapment efficiency, vesicle size, formulation of suitable gel formulae of Caffeine niosomal dispersions with penetration enhancers and others with penetration enhancers and cathodal iontophoresis, then comparing the in vitro permeation then its in vivo evaluation qualitatively by studying the effect of Caffeine on fatty tissue using histological examination and quantitatively by measuring Caffeine concentration in plasma of rats using liquid chromatography (LC–MS/MS).
**Experimental section**

**Materials**

Caffeine anhydrous was purchased from Titan Biotech Limited, India. Sorbitan monoesters (Span 20, 60, 65, 80) and Sorbitan trioleate (Span 85), Polysorbates (TWEEN 20, 60, 65, 80 and 85), and Cholesterol were kindly supplied by the Egyptian International Pharmaceutical Industries Co., (EPICO), Egypt. Chloroform was purchased from Merck KGaA, Darmstadt, Germany. Dicyclohexylphosphate (DCP), Stearylamine (SA) were purchased from Merck KGaA, Darmstadt, Germany. Carbopol 940 was purchased from Merck KGaA, Darmstadt, Germany. Propylene glycol (PG), urea, dimethylformamide (DMF), and triethanolamine were obtained from Adwic Company, Egypt. Full-thickness hairless rat skin from newly born albino rats were from animal house of Faculty of Pharmacy, Cairo University, Egypt. Commercial Product: Cellu Destock® (Caffeine pure 5% + Lipocidone) from VICHY Laboratories; France. Water for gas chromatography, HPLC and spectrophotometry manufactured by Heneywell Burdick & Jackson, Mexico City, Mexico. Dichloromethane (CHROMASOL V® for HPLC contains Amylene as stabilizer, Sigma-Aldrich).

**Methods**

**Preparation of Caffeine nonionic surfactant vesicles (niosome)**

All Caffeine nonionic surfactant vesicles (niosomes) were prepared from a mixture of nonionic surfactants, with and without cholesterol; Tables 1 and 2 using Thin Film Hydration method [21] at fixed total lipid concentration (300 μmol) and fixed drug concentration (10 ml of 2%/w/w). The calculated amount of nonionic surfactant with and without cholesterol was weighted in a rounded bottom flask then adding 15 ml chloroform prior to the attachment to the rotary evaporator for the drying process using vacuum with rotation at 60 °C. Then the dried layer is kept in the desiccator overnight to ensure complete removal of chloroform. Hydration of thin film occurs using Caffeine dissolved in deionized water for 1 h at 60 °C to form niosomal dispersion.

**Calculation of percentage of entrapment efficiency (E.E.%)**

To calculate the entrapment efficiency of Caffeine, 1 ml of niosomal dispersion was allowed to be refrigerated at 4 °C for one day, afterwards, centrifugation for 1 h at 15,000 rpm to separate the un-entrapped from niosome was done. A washing step was done to ensure that the un-entrapped drug was no longer present in the void volume between the niosomes. To the sediment, 1 ml of n-propanol was added and the mixture was shaken well for 30 min to make lysis of the niosomal vesicles to release the entrapped Caffeine; then measured using a spectrophotometer at 273 nm. Entrapment efficiency was determined as follow [23,24]:

\[
\text{Entrapment efficiency of Caffeine} = \frac{\text{Amount trapped}}{\text{Total amount of Caffeine}} \times 100
\]

All statistical differences in data of E.E.% were evaluated by IBM SPSS Statistics version 23, 64-bit edition, NY, USA, using One-Way Analysis of Variance (ANOVA) with extended LSD post hoc tests where, p values <.05 was considered significant.

**Effect of charge-inducing agents on E.E.% of Caffeine niosomes.**

The effect of charge induction on the entrapment efficiency of Caffeine niosomes was examined by using stearylamine (SA) for the induction of positive charge or dicetylphosphate (DCP) for induction of negative charge.

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**Table 1. Composition and entrapment efficiency of Caffeine niosomal dispersion using different types and ratios (%) of Span (T):Cholesterol (CHO).**

<table>
<thead>
<tr>
<th>Formula no.</th>
<th>50:50 CHO</th>
<th>60:40 CHO</th>
<th>70:30 CHO</th>
<th>80:20 CHO</th>
<th>90:10 CHO</th>
<th>E.E.%</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>1000</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>92.73 ± 0.84</td>
</tr>
<tr>
<td>F2</td>
<td>90:10</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>93.87 ± 0.67</td>
</tr>
<tr>
<td>F3</td>
<td>80:20</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>89.66 ± 0.94</td>
</tr>
<tr>
<td>F4</td>
<td>70:30</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>85.1 ± 0.76</td>
</tr>
<tr>
<td>F5</td>
<td>60:40</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>93.98 ± 1.41</td>
</tr>
<tr>
<td>F6</td>
<td>50:50</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>93.96 ± 1.03</td>
</tr>
<tr>
<td>F7</td>
<td>40:60</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>92.95 ± 0.84</td>
</tr>
<tr>
<td>F8</td>
<td>30:70</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>82.58 ± 0.72</td>
</tr>
<tr>
<td>F9</td>
<td>20:80</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>88.68 ± 0.99</td>
</tr>
<tr>
<td>F10</td>
<td>10:90</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>85.25 ± 1.19</td>
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<tr>
<td>F11</td>
<td>5:95</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>93.01 ± 0.83</td>
</tr>
<tr>
<td>F12</td>
<td>0:100</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>94.18 ± 0.75</td>
</tr>
</tbody>
</table>

**Table 2. Composition and entrapment efficiency of Caffeine niosomal dispersion using different types and ratios% of Tween (T):Cholesterol (CHO).**

<table>
<thead>
<tr>
<th>Formula no.</th>
<th>50:50 CHO</th>
<th>60:40 CHO</th>
<th>70:30 CHO</th>
<th>80:20 CHO</th>
<th>90:10 CHO</th>
<th>E.E.%</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>1000</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>93.75 ± 0.83</td>
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<tr>
<td>F2</td>
<td>90:10</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>93.87 ± 0.75</td>
</tr>
<tr>
<td>F3</td>
<td>80:20</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>90.91 ± 0.82</td>
</tr>
<tr>
<td>F4</td>
<td>70:30</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>92.06 ± 0.85</td>
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<td>F5</td>
<td>60:40</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>93.94 ± 1.03</td>
</tr>
<tr>
<td>F6</td>
<td>50:50</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>93.99 ± 1.41</td>
</tr>
<tr>
<td>F7</td>
<td>40:60</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>92.99 ± 1.01</td>
</tr>
<tr>
<td>F8</td>
<td>30:70</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>94.11 ± 0.85</td>
</tr>
<tr>
<td>F9</td>
<td>20:80</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>94.06 ± 0.83</td>
</tr>
<tr>
<td>F10</td>
<td>10:90</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>94.29 ± 1.06</td>
</tr>
<tr>
<td>F11</td>
<td>5:95</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>94.18 ± 0.75</td>
</tr>
<tr>
<td>F12</td>
<td>0:100</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>93.99 ± 1.41</td>
</tr>
</tbody>
</table>

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**DRUG DEVELOPMENT AND INDUSTRIAL PHARMACY**

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Particle size analysis, zeta (ζ)-potential measurements, and morphological examination by transmission electron microscope

The vesicular size, ζ-potential, and polydispersity index (PDI) of Caffeine niosomal dispersion were determined using dynamic light scattering with Malvern Zetasizer (Malvern Instrument Ltd., UK) [25]. Caffeine niosomal dispersion was diluted 1:10 using deionized water to avoid multi-scattering phenomena [26]. Light scattering was monitored at 25°C at a scattering angle of 90° [27]. The PDI was determined for the determination of distribution of size of niosomal vesicles [28].

Niosomes were visualized by using a transmission electron microscope (TEM, Jeol – JXA-840A – Electron Microscope – Japan) [29]. Prior to analysis, the niosomal dispersion samples were diluted 10 times with distilled water. A drop from the resultant suspension was deposited on a film-coated copper grid forming a thin liquid film. The films were then negatively stained with 2% (w/v) phosphotungstic acid solution. After air drying, the stained films were photographed by transmission electron microscopy [30].

Compatibility of Caffeine with polymer and excipient using differential scanning calorimetry (DSC)

Differential Scanning Calorimetry of Caffeine, carbopol 940 polymer, urea, and their physical mixture were performed. Samples (3–4 mg) were placed in aluminum pan and heated in the rate of 10°C/min, to a temperature of 200°C. The instrument was calibrated with indium, and dry nitrogen was used as a carrier gas with a flow rate of 25 ml/min.

Preparation of Caffeine niosomal gel with penetration enhancers (PE)

For the preparation of the plain hydrogels, appropriate amount of polymer was stirred into deionized water containing the appropriate amount of enhancer. The amounts of enhancer and polymer were adjusted to give final concentration of 5% or 10% enhancer and 1% Carbopol 940 polymer [31,32]. Few drops of triethanolamine were added to adjust the pH. For the preparation of niosomal gel bases, niosomal dispersion (F12-DCP and F39-DCP) was prepared using drug concentration (10 ml of 2% w/v), then centrifuged for 90 min at 12,000 rpm. The semisolid mass of niosomes was separated from supernatant and mixed in water before gelation of the 1% Carbopol 940 gel base. The prepared gels were packed in wide mouth glass jar covered with screw capped plastic lid (Table 4).

In vitro permeation studies of different Caffeine niosomal gel formulae with and without iontophoresis

The in vitro permeation of Caffeine niosomal gel formulae was through rat skin membrane, the permeation study was done using a dissolution-dialysis apparatus which was developed in our laboratory (Faculty of Pharmacy-Cairo University-Cairo-Egypt). The dissolution cell consists of a hollow glass cylinder (length 15 cm and internal diameter 2.52 cm). A 1 g of Caffeine niosomal gel formula is transferred to the dissolution cell to which the rat skin is attached to one side, and immersed in a receptor compartment containing 200 ml PBS (pH 5.5) stirred at 50 rpm. The temperature of the receptor compartment was maintained at temperature 32–39°C. At specified time interval for 12 h, 4 ml samples were withdrawn from the receptor compartment, each sample withdrawn was replaced by an equal volume of PBS (pH 5.5) [33]. The drug was determined spectrophotometrically at λmax 273 nm. If Cathodal Iontophoresis is to be applied during the in vitro permeation study, the cathode was dipped in the donor chamber containing 1 g of the prepared gel where the anode is placed in the receiving chamber containing PBS (pH 5.5). The current density was 0.2 mA/cm². Mode is direct continuous current for 12 h [22,34].

In vitro permeation data treatment. The in vitro skin permeation data obtained were plotted as the Q which is the cumulative amount of drug penetrated into the receptor compartment per unit area of skin membrane (µg/cm²) as a function of time (h) [35].

The responses (dependent variables) measured were:

i. The cumulative amount of caffeine passing per unit area after 12 h of start of iontophoresis, Q12 (µg/cm²).
ii. Transdermal flux (J) (µg/cm²/h), the value of the slope of the cumulative amount permeated per unit area versus time.
iii. The permeability coefficient (Kp) (ml/hr/cm²) was calculated using the equation Kp = Jp/Cv, where Cv is the total donor concentration of the solute.
iv. Enhancement factor (EF) was calculated by the ratio of the caffeine flux of the test to the caffeine flux of passive diffusion experiment

\[ \text{EF} = \frac{J_{\text{iontophoresis}}}{J_{\text{passive}}} \]

Statistical analysis of the permeation data. All data were statistically analyzed using SPSS 20, NY, USA. The mean values of Q12 were compared through one-way ANOVA, α-error value was set at 5% and the p values are considered significant if less than .05.

In vivo evaluation of Caffeine niosomal gel

Histological studies

The lipid content and water uptake properties of rat and human skin are similar, so the hairless rate skin is a good candidate for topical and transdermal permeation experiment [36].

Experiment was carried out with 24 Wister rats weighing 200–350 g split into six groups, Figure 1. All the procedures used in the present study were conducted according to the guidelines approved by the research ethics committee for experimental and clinical studies at Faculty of Pharmacy-Cairo University (Approval...
date: 21/01/2013, Code: PI670). During 21 consecutive days, for each group individually, twice a day (morning and evening), 0.5 g of the commercial gel or the prepared gels was applied (using a syringe) on the shaved dorsal skin (marked with a circle of diameter of 4 cm) of each rat. If cathodal iontophoresis is to be applied, the selected gel formulae were applied for 30 min for 21 days using Phoresor Unit II iontophoresis device (Chattanooga group, Hixson, USA) with TransQe iontophoresis electrodes of active area 13.4 cm² (OMED, Inc., Salt Lake City, UT, USA), current density was adjusted at 0.2 mA/cm², Figure 2. After asepsis of the dorsal region with ethanol, the treated area had hair removal by stainless steel blade. The six groups were categorized as:

GP I: Control.
GP II: received the commercial product Cellu Destock®.
GP III: received Caffeine niosomal gel formula FVII.
GP IV: received Caffeine niosomal gel formula FVII + iontophoresis.
GP V: received passive Caffeine niosomal gel formula FXIV.
GP VI: received Caffeine niosomal gel formula FXIV + iontophoresis.

The treatments were applied for 21 days, after which cervical dislocation of rats was done. A 2 cm² skin samples containing the fatty tissue were collected and kept for 24 h in formaldehyde to be dehydrated, then placed in paraffin [37].

Samples were fragmented vertically, the thickness of each is 5 μm, then stained with eosin and hematoxylin. Samples were placed on glass slides for examination by light microscopy [38]. Images from the fatty tissue area of each sample were captured on ×40 optic objective. These images were subjected for image analysis to determine the diameter, perimeter, and thickness of fatty layer.

**Statistical analysis**

All histological data were statistically analyzed using IBM SPSS Statistics version 23, 64-bit edition. Means for each response individually were compared by one-way ANOVA, significance level was set at \( p < .05 \).

**Quantitative evaluation by liquid chromatography (LC–MS/MS)**

Quantitative evaluation was done by mass spectrum analysis of plasma Caffeine concentration for GP II (Cellu Destock®), GP III (Caffeine niosomal gel formula FVII), and GP V (Caffeine niosomal gel formula FXIV). Rats were generally anesthetized by intraperitoneal injection of Pentobarbital (50 mg/kg). Study was designed as a parallel group design with each group containing three rats, Figure 3.

A 0.5 ml blood samples was collected via the retro-orbital vein from alternative eye at each time interval, time intervals of blood sampling were 0.5, 1, 2, 4, 8, and 12 h, after skin application into heparinized microcentrifuge tubes, the blood samples were centrifuged at 4000 rpm for 20 min to separate the plasma. The plasma samples were collected and stored at −20 °C until drug analysis. Frozen plasma samples were thawed at room temperature.

**Caffeine calibration standards extraction**

Place 150 μl plasma in a test tube then add 2 ml of (dichloromethane:diethyl ether in ratio of 30:70). Vortex the mixture for 30 s then centrifuge for 10 min. Extract the liquid layer followed by evaporation at 60 °C for 10 min. Reconstitute the dry thin film formed by 150 μl of methanol. Inject into the mass spectrum chromatograph.

**Mobile phase preparation:**

Mixture of methanol:water (80:20 v/v) + 0.1% formic acid.

**Chromatographic conditions:**

Injection volume 5 μl, flow rate 0.7 ml/min, mobile phase methanol:water (80:20), precursor ion 195, product ion 138, fragmentor 130, collision energy 15, cell accelerator voltage 8.

**Pharmacokinetic parameters calculations**

Pharmacokinetic analysis of plasma concentration of caffeine was done using pharmacokinetic add-in package for Microsoft Excel 2016 applying non-compartmental analysis. The determined pharmacokinetic parameters were maximum concentration in plasma \( (C_{\text{max}}) \), the time for maximum concentration in plasma \( (T_{\text{max}}) \), the area under plasma concentration versus time curve from zero time to 12 h \( (\text{AUC}_{0-12}) \), area under the plasma concentration versus time curve from zero to infinity \( (\text{AUC}_{0-\infty}) \), the area under momental plasma concentration versus time curve from...
zero time to 12 h (AUMC\textsubscript{0-12}), area under momental plasma concentration versus time curve from zero to infinity (AUC\textsubscript{0-\infty}), the rate constant of elimination (K\textsubscript{el}), mean residence time (MRT), and half-life (T\textsubscript{1/2}).

The experiment was pre-planned to compare the difference between the mean pharmacokinetic parameters (C\textsubscript{max}, AUC\textsubscript{0-12}, AUC\textsubscript{0-\infty}, T\textsubscript{1/2}) obtained after the administration of each of the three treatments into each group of rats in a parallel model. Analysis of variance (ANOVA) is considered the most powerful statistical tool for analyzing data from designed experiments, whose objective is to compare two or more group means.

An important question that we wish to address here is: Which treatments are different? Are all treatments different from one another, or are some treatments not significantly different? These questions may be solved using ‘multiple comparison’ procedures.

The null hypothesis is H\textsubscript{0}: \mu\textsubscript{commercial} = \mu\textsubscript{V60} = \mu\textsubscript{V64}. The alternative hypothesis is that at least two treatment means differ. The α level is set at 5%.

All statistical differences in data were evaluated by IBM SPSS Statistics version 23, 64-bit edition using ANOVA with extended LSD post hoc tests for the determined pharmacokinetic parameters where, p values <0.05 was considered significant. Non-parametric Kruskal–Wallis test was done to compare the data of T\textsubscript{max} obtained from different treatments.

**Results and discussion**

**Calculation of percentage of entrapment efficiency (E.E.%)**

Niosomes were prepared by changing the ratio % of surfactant to cholesterol while keeping both the drug concentration and the total lipid concentration constant to explore the effect of different concentrations of cholesterol on entrapment efficiency of Span and Tween series. Results are shown in Tables 1 and 2.

Niosomal vesicles (Formula 12) prepared from span 60 and CHO at a ratio 50:50 gave EE% of 94.18 ± 0.75 that is statistically significant (p values <0.05) compared to other formulae at Table 1, for Span Caffeine niosomes were prepared using span 20, span 60, and span 65, the entrapment efficiency of Caffeine is directly proportional to cholesterol concentration. There was an almost direct proportionality between entrapment efficiency and cholesterol content up to 50% [39]. The 1:1 ratio between the cholesterol and nonionic surfactant in the bi-layers shows an optimum ratio for the production of physically stable niosomal systems [40], this could be attributed to an increase in the vesicle size by cholesterol [41].

Span 60 is solid at room temperature and showed the higher phase transition temperatures (T\textsubscript{c}, °C) about 55 °C compared to other spans [42], and it produced niosome with high entrapment efficiency of flubiprofen than other spans [22].

Presence of cholesterol has its impact on stability and permeability of the prepared niosomal vesicles [43]. Cholesterol increases the hydrodynamic diameter and entrapment efficiency of niosomes. Incorporation of cholesterol increases the bilayer hydrophobicity and stability of niosomes, reducing the permeability [44].

Niosomal vesicles (Formula 39) prepared from Tween 60 and CHO at a ratio 80:20 gave EE% of 94.29 ± 1.06 that is statistically significant (p values <0.05) compared to other formulae at Table 2, for all Tween, prepared Caffeine niosomes, the entrapment efficiency of Caffeine increased as concentration of cholesterol increased up to 20% further increase in cholesterol concentration result in a slight decrease. The niosomes prepared without cholesterol had certain E.E.% and this value was increased with increasing the concentration of cholesterol from 0% to 50%, this could be attributed to an increase in the vesicle size by cholesterol [22]. Increasing cholesterol ratio above certain limit lead to decrease of the entrapment efficiency by disrupting the regular bilayer structure of vesicular membrane leading to the loss of encapsulated drug [45,46]. The larger size could offer the high E.E. values as it is usually recognized that it significantly increased with particles size [15].

Tween 60 compared to other surfactants was found to give the highest E.E.% of a water soluble local antibiotic Gentamicin Sulphate [47]. The surfactant having a higher phase transition temperature (T\textsubscript{c}, °C) produces niosome with the highest entrapment efficiency [48].

Regarding Caffeine niosomes prepared with Span 60 or Tween 60 at ratio of 47.5:47.5:5 for Span60:cholesterol:charge inducer (+), Table 3, the negatively charged Caffeine niosomes exhibited the highest entrapment efficiency followed by neutral Caffeine niosomes and then the positively charged (F12-DCP > F12-SA) and (39-DCP > F39 > F39-SA), this result is consistent with the weak basic nature of caffeine so an electrostatic attraction would occur between drug cation and negatively charged DCP, inclusion of DCP also results in marked repulsion between vesicle interfaces and hence an increase in the distance between adjacent bilayers that will prevent vesicle aggregation [34,49-51].

**Particle size analysis, zeta (\zeta)-potential measurements, and morphological examination by transmission electron microscope**

The niosomes were identified with perfect lipid bilayer either spherical or elongated niosomes using the Transmission Electron Microscopy images. The shape differs according to the type of
surfactant; Span-formed niosomes are spherical in shape while Tween-formed niosomes are elongated, Figure 4(a and b).

Table 5 shows the mean size (nm), polydispersity index (PDI), and \( \zeta \)-potential (mV) of some representative Caffeine niosomal dispersions.

<table>
<thead>
<tr>
<th>Formula</th>
<th>Surfactant</th>
<th>HLB</th>
<th>Mean size (nm)</th>
<th>PDI</th>
<th>( \zeta )-potential (mV)</th>
</tr>
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<tbody>
<tr>
<td>F6</td>
<td>( S_{50} )</td>
<td>8.6</td>
<td>380</td>
<td>0.624</td>
<td>–66</td>
</tr>
<tr>
<td>F12</td>
<td>( S_{60} )</td>
<td>4.7</td>
<td>274</td>
<td>0.399</td>
<td>–34.6</td>
</tr>
<tr>
<td>F18</td>
<td>( S_{65} )</td>
<td>2.1</td>
<td>183.9</td>
<td>0.337</td>
<td>–19</td>
</tr>
<tr>
<td>F24</td>
<td>( S_{65} )</td>
<td>1.8</td>
<td>174</td>
<td>0.874</td>
<td>–21.9</td>
</tr>
<tr>
<td>F36</td>
<td>( T_{20} )</td>
<td>16.7</td>
<td>1069</td>
<td>0.917</td>
<td>–27.7</td>
</tr>
<tr>
<td>F39</td>
<td>( T_{60} )</td>
<td>14.9</td>
<td>641</td>
<td>0.431</td>
<td>–37.2</td>
</tr>
<tr>
<td>F48</td>
<td>( T_{65} )</td>
<td>10.5</td>
<td>441</td>
<td>0.388</td>
<td>–22.3</td>
</tr>
<tr>
<td>F54</td>
<td>( T_{65} )</td>
<td>15</td>
<td>996</td>
<td>0.494</td>
<td>–29.7</td>
</tr>
<tr>
<td>F60</td>
<td>( T_{80} )</td>
<td>11</td>
<td>470.8</td>
<td>0.733</td>
<td>–31.8</td>
</tr>
</tbody>
</table>

Niosomes vesicles having values of \( \zeta \)-potential around \(-30\) mV are more stable due to presence of electrical repulsion, the higher the value of \( \zeta \)-potential, the more stable the niosomal vesicles [28].

**Compatibility of Caffeine with Carbopol 940 polymer and excipients in the prepared gel using DSC**

The thermograms for pure Caffeine, Carbopol 940, urea, and the physical mixture of Caffeine, Carbopol, and urea at 1:1:1 ratio were determined using DSC calorimeter, Figure 6. Caffeine exhibited a sharp endothermic peak around 240°C (melting point), no sharp peak was observed at carbopol 940 thermogram, urea exhibited its characteristic peak around 150°C. Physical mixture of caffeine, urea, and carbopol 940 showed the characteristic peak of caffeine at 240°C, revealing no interaction between the components in the mixture.

**In vitro permeation studies of different Caffeine niosomal gel formulae with and without iontophoresis**

The permeation studies were conducted in two conditions, either by applying chemical penetration enhancer on the formulae alone or in combination with iontophoresis. The presence of penetration enhancers with iontophoresis increased \( Q \) of Caffeine from the prepared gel formulae, Figure 7(a and b).

The permeation data as \( Q_{12} \), flux, enhancement factor (E.F.), and permeability coefficient (Kp) for the formulae are shown in Table 6.

The Caffeine niosomal gel formulae with (2% w/w) drug concentration were subjected to permeation studies using full-thickness hairless rat skin and a continuous Direct Continuous Current of 0.2 mA/cm² for 12 h. There is a synergistic effect between bilayer disruptor (chemical enhancer) and a driving force provider (iontophoresis) due to effect of penetration enhancer on widening intracellular spaces in
Figure 5. Particle size of Caffeine niosomes prepared (a) using S60 (F12) and (b) using T60 (F39).

Figure 6. DSC thermogram for Caffeine, urea, carbopol 940 and their physical mixture (1:1:1).

Figure 7. Effect of penetration enhancers and cathodal iontophoresis on the permeation of Caffeine from (a) different formulae (FI-FVII) drug niosomal gel, (b) different formulae of (FVIII-FXIV) drug niosomal gel through hairless rat skin (n = 3).

Table 6. The permeation data of different caffeine niosomal gel formulae containing penetration enhancer with iontophoresis through hairless rat skin.

<table>
<thead>
<tr>
<th>Formula</th>
<th>Flux, J (μg h/cm²)±SD</th>
<th>Q12 (μg/cm²)±SD</th>
<th>Enhancement Factor (EF)±SD</th>
<th>Permeability coefficient, Kp (ml h/cm²)±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>FI</td>
<td>66.41 ± 0.930</td>
<td>4486.95 ± 62.82</td>
<td>1.851 ± 0.026</td>
<td>0.040 ± 0.0006</td>
</tr>
<tr>
<td>FII</td>
<td>122.94 ± 2.09</td>
<td>5562.17 ± 94.56</td>
<td>2.332 ± 0.07</td>
<td>0.101 ± 0.0017</td>
</tr>
<tr>
<td>FIII</td>
<td>148.21 ± 2.06</td>
<td>7250.90 ± 30.52</td>
<td>3.753 ± 0.08</td>
<td>0.165 ± 0.0027</td>
</tr>
<tr>
<td>FIV</td>
<td>249.22 ± 3.49</td>
<td>6357.83 ± 89.01</td>
<td>6.273 ± 0.09</td>
<td>0.225 ± 0.0027</td>
</tr>
<tr>
<td>FV</td>
<td>401.41 ± 4.42</td>
<td>8373.89 ± 12.11</td>
<td>9.608 ± 0.10</td>
<td>0.295 ± 0.0033</td>
</tr>
<tr>
<td>FVI</td>
<td>382.4 ± 6.501</td>
<td>7267.46 ± 13.53</td>
<td>6.493 ± 0.09</td>
<td>0.23 ± 0.0039</td>
</tr>
<tr>
<td>FVII</td>
<td>568.83 ± 11.38</td>
<td>8875.30 ± 177.51</td>
<td>8.73 ± 0.12</td>
<td>0.342 ± 0.0068</td>
</tr>
<tr>
<td>FVIII</td>
<td>49.47 ± 0.69</td>
<td>3835 ± 53.69</td>
<td>3.98 ± 0.05</td>
<td>0.030 ± 0.0004</td>
</tr>
<tr>
<td>FIX</td>
<td>197.09 ± 3.35</td>
<td>3568.87 ± 60.67</td>
<td>4.34 ± 0.07</td>
<td>0.120 ± 0.002</td>
</tr>
<tr>
<td>FX</td>
<td>214.86 ± 4.30</td>
<td>4431.12 ± 88.62</td>
<td>7.05 ± 0.10</td>
<td>0.165 ± 0.0023</td>
</tr>
<tr>
<td>FXI</td>
<td>270.64 ± 3.79</td>
<td>4984.95 ± 69.79</td>
<td>9.78 ± 0.13</td>
<td>0.296 ± 0.0033</td>
</tr>
<tr>
<td>FXII</td>
<td>483.77 ± 5.32</td>
<td>5532.46 ± 60.86</td>
<td>9.95 ± 0.11</td>
<td>0.298 ± 0.0051</td>
</tr>
<tr>
<td>FXIII</td>
<td>492.52 ± 8.56</td>
<td>5206.95 ± 88.52</td>
<td>10.18 ± 0.11</td>
<td>0.30 ± 0.0051</td>
</tr>
<tr>
<td>FXIV</td>
<td>503.52 ± 8.56</td>
<td>5648.81 ± 116.98</td>
<td>10.18 ± 0.11</td>
<td>0.30 ± 0.0051</td>
</tr>
</tbody>
</table>

Each result is the mean of 3 determinations ± SD.
Figure 8. Photomicrograph of skin (H&E ×40) from (a) control rat showing large diameter & abundant number of fat cells, (b) rat treated with commercial product formula Cellu Destock®, (c) rat treated with formula FVII, (d) rat treated with formula FXIV, (e) rat treated with formula FVII + Ionto, and (f) rat treated with formula FXIV + Ionto (all photos have the same magnification scale as shown at (a)).
skin, which in turn will make iontophoresis more influential [55]. These results are in accordance with the studied synergism in the transdermal delivery of tolterodine using PE and iontophoresis [56], synergism between niosomal formulation and iontophoresis in improving the transdermal permeation profile of isosorbide dinitrate [57] and the synergistic effect of iontophoresis and PE in the permeation of triamcinolone during investigation of the carbopol gel of solid lipid nanoparticles [58].

Incorporation of PG increased the transdermal delivery of caffeine, these results are consistent with the study of the transdermal skin penetration of lornoxicam into the skin layers that was improved by presence of PG [59], the enhanced transdermal flux of Zidovudine (AZT) by about 200-fold due to presence of PG and oleic acid, and its enhancement by 7-fold due to application of iontophoresis alone [60]. Propylene glycol (PG) was employed to clonazepam gel in Carbopol 934, drug permeation progressively increased by increasing enhancer content in the vehicle, PG acts as co-solvents promoting the drug release from the dosage form by increasing the solubility and therefore the concentration gradient of the drug in the gel [61]. Dimethylformamide (DMF) dissolves the shell around the polar groups of lipid, makes the lipid more fluidized, and disrupts its packing and partition to keratin layers thus acting as efficient penetration enhancer [62,63].

### Table 7. Effect of different formulae on different parameters of fat cell such as diameter, area and thickness of fat layer.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Cellu Destock®</th>
<th>FVII</th>
<th>FVII + Iontophoresis</th>
<th>FVIX</th>
<th>FVIX + Iontophoresis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radius (μm)</td>
<td>9.615 ± 0.495</td>
<td>5.59 ± 0.286</td>
<td>4.375 ± 0.658</td>
<td>6.29 ± 0.286</td>
<td>5.245 ± 0.665</td>
<td>6.025 ± 0.202</td>
</tr>
<tr>
<td>Diameter (μm)</td>
<td>19.23 ± 0.99</td>
<td>11.18 ± 0.572</td>
<td>8.75 ± 1.315</td>
<td>12.58 ± 0.572</td>
<td>10.49 ± 1.330</td>
<td>11.05 ± 0.404</td>
</tr>
<tr>
<td>Perimeter (μm)</td>
<td>60.415 ± 3.107</td>
<td>35.15 ± 1.796</td>
<td>27.46 ± 4.160</td>
<td>39.54 ± 1.792</td>
<td>33.043 ± 4.189</td>
<td>38.375 ± 1.273</td>
</tr>
<tr>
<td>Area (μm²)</td>
<td>290.5 ± 30.96</td>
<td>104.66 ± 11.802</td>
<td>60.11 ± 20.413</td>
<td>124.44 ± 11.29</td>
<td>86.8 ± 20.737</td>
<td>99.89 ± 5.104</td>
</tr>
<tr>
<td>Thickness (μm)</td>
<td>183.23 ± 11.37</td>
<td>66.83 ± 19.513</td>
<td>33.225 ± 9.397</td>
<td>54.27 ± 9.928</td>
<td>58.05 ± 21.51</td>
<td>63.29 ± 5.947</td>
</tr>
<tr>
<td>% decrease in the area</td>
<td>–</td>
<td>63.97</td>
<td>79.32</td>
<td>57.16</td>
<td>70.12</td>
<td>51.48</td>
</tr>
<tr>
<td>% decrease in the thickness</td>
<td>–</td>
<td>63.52</td>
<td>81.86</td>
<td>70.38</td>
<td>68.31</td>
<td>65.45</td>
</tr>
</tbody>
</table>

Figure 9. Effect of different treatments on different parameters of fat cell such as diameter, area, and thickness of fat layer.

### In vivo evaluation of Caffeine niosomal gel

#### Histological studies

The normal histological structure of the dorsal region of rat skin (Control) showing abundant number of large diameter fat cells, Figure 8(a).

Table 7 and Figure 8(b–f) illustrate the fatty cells appearance after the treatment with the commercial product, (FVII), (FXIV), (FVII + Iontophoresis), and (FXIV + Iontophoresis) respectively. Different Caffeine niosomal gel formulae caused a reduction of (51.48–79.3%) on the area of fatty layer compared with the control (untreated with Caffeine) (see Figure 9).

Concluding from the image analysis of previous figures; that the best Formula is FVII, followed by FXIV, followed by commercial...
product, followed by FVII + Iontophoresis and finally, FXIV + Iontophoresis. This may be attributed to the residence time which was greater in FVII, followed by FXIV which contains penetration enhancer, followed by FVII + Iontophoresis and FXIV + Iontophoresis; the last two contain penetration enhancer and applying Iontophoresis, which may result in minimizing the residence time of the drug on the hypodermis.

All prepared formulae showed a significant decrease in the thickness of fat layer compared with control. Ranking of the % decrease in the thickness was FVII > FXIV > Cellu Destock® > FVII + Iontophoresis > FXIV + Iontophoresis.

Quantitative evaluation of Caffeine in plasma by Liquid Chromatography (LC–MS/MS):

Determination of the concentration of caffeine in plasma after its passing through the rat skin was done. Calibration curve was constructed using different concentration of Caffeine. A sample of the LC–MS/MS chromatograms of caffeine in plasma is presented in Figure 10.
The mean Caffeine plasma level in rats after application of Cell Destock\(^{\text{V}}\), formula FVII and formula FXIV versus time was computed and the data are presented in Figure 11.

The pharmacokinetics parameters of different formula of Caffeine as \(C_{\text{max}}\), \(T_{\text{max}}\), \(AUC_{0-12}\), \(AUC_{0-\infty}\), \(AUMC_{0-12}\), \(AUMC_{0-\infty}\), MRT, \(K_{el}\), and \(T_{1/2}\) were calculated and are presented in Table 8.

Results revealed that after topical application of FVII, FXIV, and commercial Cell Destock\(^{\text{V}}\) to rats, drug gave maximum appearance in plasma after a \(4.67 \pm 0.3055\) h, \(4.67 \pm 1.1547\) h, and \(1.167 \pm 0.76376\) h respectively. Mean peak drug concentration \(C_{\text{max}}\) \((9.589053 \pm 2.34598 \, \text{mg/ml})\) was higher than that of FVII \((1.18 \pm 0.21377 \, \text{mg/ml})\) and market product \((0.67244 \pm 0.07254 \, \text{mg/ml})\). The mean time to reach the peak concentration \((t_{\text{max}})\) was comparable, that no statistically significant difference \((p > .05)\) was observed among the \(t_{\text{max}}\) values of three treatments.

Moreover, the \(AUC_{0-12}\) value was \(4.37646 \pm 0.97234\), \(6.524128 \pm 0.9134\), and \(38.15249 \pm 2.8792 \, (\text{mg h ml}^{-1})\) for commercial Cell Destock\(^{\text{V}}\) product, FVII, and FXIV, respectively. The \(AUC_{0-\infty}\) value

![Figure 11. Plasma level of Caffeine following application of commercial Cellu Destock\(^{\text{V}}\), FVII, and FXIV into rat skin (results are average of three rats, ±SD).](image)

<table>
<thead>
<tr>
<th>Pharmacokinetic parameters</th>
<th>Cellu Destock(^{\text{V}})</th>
<th>FVII</th>
<th>FXIV</th>
</tr>
</thead>
<tbody>
<tr>
<td>(C_{\text{max}}) (\text{mg/ml})</td>
<td>(0.672 \pm 0.072)</td>
<td>(1.180 \pm 0.213)</td>
<td>(9.589 \pm 2.345)</td>
</tr>
<tr>
<td>(T_{\text{max}}) (h)</td>
<td>(1.167 \pm 0.763)</td>
<td>(4.670 \pm 3.055)</td>
<td>(4.670 \pm 1.1547)</td>
</tr>
<tr>
<td>(AUC_{0-12}) (\text{mg h/ml})</td>
<td>(4.376 \pm 0.972)</td>
<td>(6.524 \pm 0.913)</td>
<td>(38.152 \pm 2.879)</td>
</tr>
<tr>
<td>(AUC_{0-\infty}) (\text{mg h/ml})</td>
<td>(7.538 \pm 0.625)</td>
<td>(7.450 \pm 0.782)</td>
<td>(39.745 \pm 3.024)</td>
</tr>
<tr>
<td>(AUMC_{0-12}) (\text{mg h}^2/ml)</td>
<td>(22.770 \pm 1.852)</td>
<td>(29.934 \pm 7.657)</td>
<td>(150.846 \pm 9.704)</td>
</tr>
<tr>
<td>(AUMC_{0-\infty}) (\text{mg h}^2/ml)</td>
<td>(60.718 \pm 4.993)</td>
<td>(41.049 \pm 3.847)</td>
<td>(169.960 \pm 19.735)</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>(6.0541 \pm 0.545)</td>
<td>(5.19 \pm 0.853)</td>
<td>(4.247 \pm 0.649)</td>
</tr>
<tr>
<td>(K_{el}) (h(^{-1}))</td>
<td>(0.176 \pm 0.035)</td>
<td>(0.219 \pm 0.031)</td>
<td>(0.354 \pm 0.051)</td>
</tr>
<tr>
<td>(T_{1/2}) (h)</td>
<td>(3.934 \pm 0.274)</td>
<td>(3.154 \pm 0.244)</td>
<td>(1.957 \pm 0.212)</td>
</tr>
</tbody>
</table>

Table 8. The pharmacokinetic parameters of Caffeine obtained after application of commercial Cellu Destock\(^{\text{V}}\), FVII, and FXIV into rat skin (results are average of three rats, ±SD).

<table>
<thead>
<tr>
<th>Statistical test</th>
<th>One-way ANOVA</th>
<th>Independent samples Kruskal–Wallis test of (T_{\text{max}}) and the alternative hypothesis here is that at least two treatment means differ across different treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Null hypothesis</td>
<td>(H_0^{\text{V}}: h_{\text{commercial}} = h_{\text{FVII}} = h_{\text{FXIV}})</td>
<td>The distribution of (T_{\text{max}}) is the same across different treatments</td>
</tr>
<tr>
<td>(p) value (the significance level is .05)</td>
<td>(C_{\text{max}})</td>
<td>(AUC_{0-12})</td>
</tr>
<tr>
<td>Commercial Cellu Destock(^{\text{V}})</td>
<td>FVII</td>
<td>0.571</td>
</tr>
<tr>
<td>FVII</td>
<td>FXIV</td>
<td>0.00</td>
</tr>
<tr>
<td>FXIV</td>
<td>Commercial Cellu Destock(^{\text{V}})</td>
<td>0.00</td>
</tr>
<tr>
<td>FVII</td>
<td>Commercial Cellu Destock(^{\text{V}})</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Table 9. ANOVA table of pharmacokinetic parameters of caffeine following application of commercial Cellu Destock\(^{\text{V}}\), FVII, and FXIV into rat skin \((n = 3)\).
respectively. The AUMC \(0 \pm 1.04958 \pm 3.84705\) and \(169.9609 \pm 19.73583\) for commercial Cell Destock\textsuperscript{0} product, FVII, and FXIV, respectively. Moreover, the AUMC\textsubscript{0–12} value was 22.77074 \pm 1.85266, 29.93432 \pm 7.6576, and 150.8466 \pm 9.70457 (\(\mu g\) ml\(^{-1}\)) for commercial Cell Destock\textsuperscript{0} product, FVII, and FXIV, respectively. The AUMC\textsubscript{0–inf} value was 60.71868 \pm 4.99373, 41.04958 \pm 3.84705, and 169.9609 \pm 19.73583 (\(\mu g\) ml\(^{-1}\)) for commercial Cell Destock\textsuperscript{0} product, FVII, and FXIV, respectively, suggest that commercial Cell Destock\textsuperscript{0} product showed the lowest rate and extent of drug absorption, whereas, FXIV showed the highest rate and extent of drug absorption.

Analysis of variance applied to log \(C_{\text{max}}\) log \(AUC_{0–12}\), log \(AUC_{0–inf}\), log \(AUMC_{0–12}\), log \(AUMC_{0–inf}\), and \(T_{\text{max}}\) data are shown in Table 9. There are statistical significant differences between the values of log \(C_{\text{max}}\) log \(AUC_{0–12}\), log \(AUC_{0–inf}\), log \(AUMC_{0–12}\), log \(AUMC_{0–inf}\) calculated for FXIV and FVII and calculated for FXIV and commercial Cell Destock\textsuperscript{0} product.

The combined results from the histological study and the pharmacokinetic study may be explained depending on the fact that, cellulite is a dermal disturbance in fatty tissue, so the treatment that keeps the drug in the dermis will give the most benefit for the treatment of cellulite. Use of iontophoresis with PE increased the transdermal penetration of caffeine thus its appearance in circulation with a greater amount (higher \(C_{\text{max}}\) and AUC), but its presence in dermis is less so the anticellulite activity is reduced.

**Conclusions**

In this study, niosomal vesicles were prepared using film hydration technique then the best niosomal dispersions were formulated into a gel with different penetration enhancers. From this study, it was concluded that the prepared niosomal vesicles were with high caffeine entrapment efficiency and having globule size in the nanometric range which may be physiologically stable. It was also concluded that prepared gel with penetration enhancers and iontophoresis gave a faster \textit{in vitro} permeation through rat skin than the simple diffusion only. In conclusion, niosomal vesicles-containing gel drug delivery systems represented a promising approach for the formulation of transdermal anti-cellulite product of caffeine.

Finally, the transdermal delivery of anti-cellulite drugs can be made possible by niosomal gels, which have been shown to substantially improve the transdermal bioavailability.

**Disclosure statement**

Mahmoud H. Teaima, Sally A. Abdelhalim, Mohamed A. El-Nabarawi, Dalia A. Attia, and Doaa A. Helal declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

**References**


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