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# Formulation Development and Evaluation of Aspasomes Containing Skin Whitening Agent

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## Abstract

Skin darkening in elderly and middle age men and women has been a constant problem which is both aesthetic as may also be pathological in nature. Demand for skin lightening cosmetics is immense, as many people wish to modify, change, their skin colour, or for depigmenting skin in treatment of hyperpigmentation, freckles, or lentigines. The major enzyme that is involved in the melanin synthesis is Tyrosinase and skin whitening may occur in the event of the use of Tyrosine inhibitors which decrease or block the synthesis of melanin. For the treatment of skin whitening, targeted biological vesicles may be used. In the present research, cholesterol and negatively charged lipids were used along with Ascorbyl palmitate (ASP) and Quercetin (QZT) to formulate bilayer vesicles. When the lipid formed the aspasomes, then Quercetin was encapsulated in the bilayer by sonication. It was observed that when a combination of ascorbyl palmitate and Quercetin was used, a synergistic effect was observed and could be used as a skin lightening cosmetic product. Quercetin inhibits tyrosinase formation inhibiting melanin formation and ascorbyl palmitate has antioxidant and skin whitening property and is carrier. The formulations were optimized for particle size and per cent drug entrapment at different temperature, volume of hydration, and time of hydration. The content of Ascorbyl palmitate and cholesterol used had an effect on the size and entrapment in the vesicles. Franz diffusion assembly was used to study the in-vitro transdermal permeation of aspasomal QZT, aspasomal-QZT gel and QZT-solution across excised rat skin and it was found that the results were best with the aspasomal QZT.

**Key words:** Ascorbyl palmitate, Skin whitening agent, Quercetin, vesicles, Transdermal penetration

## Introduction

The demand for skin lightening cosmetics is immense, as many people wish to modify, or change, their skin colour, whilst others use it for de-pigmenting the skin in the treatment of hyper-pigmentation, freckles, or lentigines. Melanin defends the harmful effects of ultra violet light of the sun and therefore skin lightening may be achieved by blocking or somehow reducing the effect of tyrosinase inhibitors. Tyrosinase is a glycosylated, monooxygenase containing copper, which is associated with many functions in our body. It is widely available in nature. Primarily, this

enzyme is associated with the initial two steps in the melogenesis in mammals which is the process of production of melanin (a dark macromolecule) and is responsible to give a certain colour to hairs and skin of mammals. Hyper-pigmentation occurs upon the over-activity of this enzyme because of the overproduction of melanin. When the enzyme tyrosinase is inhibited, it leads to reduced melanin production.

The amino acid L-tyrosine undergoes a two-step hydroxylation to form L-3,4-dihydroxyphenylalanine, L-DOPA, and then the oxidation of L-DOPA occurs to form dopaquinone<sup>1</sup>. This quinone is a highly reactive compound and can spontaneously polymerize to form melanin. Therefore, the skin whitening activity is usually studied through the inhibition ability of dopachrome formation.

Ascorbic acid and various derivatives of it have been used in the preparation of dermatological and

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cosmetic formulations. As it has the capability to reduce skin pigmentation and melanin decomposition and also improves skin elasticity by aiding collagen formation hence finds its use in skin whitening products. Ascorbic acid is less stable than Ascorbyl palmitate. The synthetic lighteners may cause skin sensitization and also show cytotoxic side effects. Therefore, safer alternatives containing Quercetin, and Vitamin C and its derivatives are useful and show synergistic effects. Also, these do not have cytotoxic effects to melanocytes. Quercetin is a potent inhibitor of melanogenesis and is a flavonoid by nature. It also decreases the intracellular tyrosinase activity. The inhibition of melanogenesis by quercetin is found to be due to both its tyrosinase inhibition activity along with protein expression.

Quercetin is been found to be associated with either inhibiting the biosynthesis of melanin biosynthesis or cause for inhibition of free-radical formation. It enhances collagen biosynthesis. But due to its low chemical stability, the practical applications are limited. When Ascorbyl palmitate is used along with Quercetin, better skin absorption, especially as a lipid based delivery system for topical delivery, is observed. Aspasomes are lipophilic drug carrier systems and can act as a local depot to improve drug action for skin whitening and anti-ageing effects for different classes of drugs.

## Materials and Methods

Quercetin was obtained from Hi Media Laboratories, Mumbai, India. Ascorbic acid-6-palmitate was obtained from Sigma Chemicals, St Louis, M O, USA; and Cholesterol from Loba Chemie Pvt Ltd.

Mumbai and Span 40 was obtained from SD fine-Chemicals Ltd, Mumbai. All other chemicals used were of analytical grade.

### Preparation of Aspasomes

Film hydration was used to prepare aspasomes under nitrogen atmosphere, where the lipid mixture (ascorbyl palmitate: cholesterol) in molar ratio (Table 1) was mixed with Span 40. The drug was added to a mixture of chloroform and methanol and this system was kept under reduced pressure in rotary evaporator at a temperature of 50 °C to form a thin dry film on wall of round bottom flask. Then, 10 ml of phosphate buffered saline (PBS pH 7.4) for hydration of the dried thin lipid film was added. Ultrasonication was done for 2 minutes at 50% of maximum output and then aspasomes were stored in nitrogen-purged vials.

### Preparation of Aspasomal-gel

Carbapol gel was added to the Aspasomes dispersion in ratio of 1:1 (w/w) at 200 rpm/min for 5 minutes. Various concentrations of Quercetin and carbapol were prepared and parabens along with 2–15% of propylene glycol were added to the Aspasomal gels.

## Characterization

### Characterization of Aspasomes

#### Photomicrography

The aspasomes (unsonicated) were photomicrographed using a camera fitted over microscope to confirm formation and pattern/nature of vesicles.

**Table 1: Formulation Codes of ASP Formulations**

S No	Ingredients (mol %)	Formulations						
		ASP1	ASP2	ASP3	ASP4	ASP5	ASP6	ASP7
1.	Ascorbyl palmitate	50	55	45	40	60	75	25
2.	Cholesterol	50	45	55	60	40	25	75
3.	Drug	20 mg						
4.	Chloroform and methanol (9:1)	qs (As minimum quantity to dissolve Ascorbyl palmitate: cholesterol mixture).						
5.	Phosphate buffer (pH 7.4)	qs (To make up volume up to 10 ml).						
<b>For gel</b>								
1.	Carbopol-940	0.5-2.5 % w/w						
2.	Propylene glycol	15						
3.	Methyl paraben	0.2						
4.	Triethanolamine	To adjust pH						
5.	DM water	qs						

### Size and size distribution

Aspasomal particle size and particle size distribution was determined using a dynamic light scattering system (Malvern 4700, Malvern, UK), where the temperature was 90° and He-Ne laser system was used.

### Per cent entrapment

The Quercetin entrapment capacity was done by centrifugation using a cooling micro centrifuge. The specifications were 4 °C at 12,000 rpm for 15 minutes. The supernatant (free drug in PBS pH7.4) and sediment (vesicle entrapping the Quercetin) were collected separately. The supernatant and sediment (lysis of vesicles by Triton X-100 0.5% v/v or methanol) were removed and analyzed for drug.

The entrapment efficiency was calculated using:

$$EE \% = [(T-S)/ T] \times 100$$

Where,

T is the total amount detected both in supernatant and sediment.

S is the amount detected only in the supernatant.

### Surface Morphology (SEM)

The SEM images were observed using Hitachi S-576 Scanning electron micrograph at a temperature of 25 ± 2°C. For this, appropriate dilutions of aspasomal dispersion were made and sonicated. A few drops of formulation were placed on the grid and dried. After drying, the photographs were taken.

### Zeta potential

Malvern Zetasizer 2000 was used to measure zeta potential of the formulations. The dispersion was mixed with the PBS pH 7.4 and measurements were taken in triplicate in multimodal mode. The charge on vesicles and mean zeta potential values were directly obtained.

### Characterization of Aspasomal gel

#### Viscosity

Viscosity of gels was measured using Brookfield digital viscometer. The TF S-96 spindle at 10 rpm was used. Once gel settled at room temperature, the measurements were taken.

**Table 2: Formulation and physicochemical characterization of quercetin aspasomes**

Codes	Particle size (nm)	Percent Entrapment Efficiency (%)	Zeta potential (mv)	
ASP 1	384.2±5.48	52.41±0.60	-48.2±2.4	
ASP 2	414.8±6.42	46.72±0.86	-45.3±2.8	
ASP 3	438.6±7.36	33.82±1.46	-42.4±5.4	
ASP 4	498.8±7.64	27.42±1.20	-39.8±4.4	
ASP 5	680.2±6.10	30.80±1.68	-36.2±3.3	
ASP 6	632.7±5.84	25.56±2.80	-31.3±4.2	
ASP 7	768.3±10.4	17.42±2.48	-27.6±3.8	
Of Gel				
	Spreadability (g.cm/s)	Viscosity (cps±SD)	pH value	Drug content
APG 1	32.42	33480±2.54	7.1	91.4
APG 2	24.28	37520±3.20	6.8	89.5
APG 3	16.38	43780±1.82	6.7	87.3
APG 4	9.44	50220±3.48	6.4	88.3
APG 5	5.62	54220±2.20	6.3	82.2

### Spreadability

A wooden block and glass was used to determine spreadability. A wooden block was fixed with a pulley and glass slide and a pan was attached. Measured amount of the gel was placed on fixed glass slide so that the gel was exactly between the two slides for a duration of 5 minutes. The total time taken for the slides to separate was noted. The experiment was done in triplicate and the results noted.

Spreadability was determined using formula:

$$S=M.L/T$$

Where,

S is spreadability in gm-cm/sec,

M is mass in grams,

L is glass length and

T is time in seconds.

### Extrudability

Extrudability was determined by finding out the amount of gel extruded from the lacquered aluminium collapsible tube containing the gel. Upon removal of the cap, the gel extruded out until pressure dissipated. The weight of 0.5 cm ribbon

of the extruded aspasomal-gel in 10 seconds was determined using the formula”

$$E = (W_e / W_t) \times 100$$

Where,

E = % Extrudability

$W_e$  = Amount of gel extruded in g

$W_t$  = Total amount of gel in the tube in g

### Measurement of pH

Digital pH meter at a constant temperature was used to measure pH of the gel.

### Drug content

One gram of Aspasomal gel was diluted with 10 ml of distilled water containing Triton X-100 (0.5% v/v) to cause lysis of vesicles. It was mixed to get a slightly opaque dispersion and the centrifuged at 3,000 rpm for 30 minutes. The supernatant was filtered through the polycarbonate membrane filter (0.20  $\mu$ m) and diluted with 10 ml of distilled water and analyzed for drug. The Quercetin concentration was determined by measuring absorbance at 369 nm using UV-spectrophotometer.

### Comparative evaluation of Developed system and Aspasomal gel

#### *In vitro* release study

Release studies were carried out after removing un-entrapped Quercetin by dialyzing the original preparation. A dialysis membrane (Hi-Media, Mumbai) weight cut off between 12,000 and 14,000 daltons was used. Pre-soaked membrane (phosphate buffer pH 7.4 for 12 hours) was used and 5 ml of dialyzed preparation was added and release studies carried out taking 100 ml of PBS 7.4 in receptor cell. Aliquots (5 ml) were withdrawn at regular intervals and refreshed with fresh buffer. The

study was carried out for 18 hours. Samples were analyzed for Quercetin using Ultraviolet (UV) spectrophotometer. The same was repeated with aqueous Quercetin solution and aspasomal-gel. All experiments were performed in triplicate and reported as mean  $\pm$  standard deviation.

#### *In vitro* Transdermal permeation

The in-vitro permeation of the formulations was studied using Franz diffusion assembly at a temperature of  $32 \pm 1^\circ\text{C}$ . Phosphate buffer saline pH 6.4 (16 ml) was placed in the receptor compartment and stirred at 100 rpm using a magnetic stirrer. The dorsal skin was excised of the sacrificed rat and kept in distilled water initially, then placed on aluminium foil and the adhering fat and/or subcutaneous tissue from the dermal side of skin were gently removed. This skin was mounted onto the receptor compartment with the stratum corneum side facing upward into the donor compartment and the aspasomal formulation (200  $\mu$ l) was applied.

One ml of aliquots were withdrawn through the sampling port at predetermined time intervals over 24 hours, replenished with equal volume of fresh buffer and analyzed for drug content. The experiments were performed in triplicates. Same were performed with aqueous Quercetin solution, and aspasomal-gel and the cumulative amount of drug permeated determined.

#### *In vivo* study

##### Depigmenting Activity

Depigmenting activity was evaluated in mice. Five week old mice were selected with weights ranging from 20 gms-25 gms and housed in stainless steel cages (temperature 25-28 $^\circ\text{C}$ ) and with alternating day and night cycles of 12 hours. The animals were allowed to adjust for 7 days prior to the experiment. The hairs were shaven and then the animals were

**Table 3: Stability studies of ASP-QZT dispersion and ASP-QZT gel**

Number of days	ASP-quercetin dispersion				ASP-quercetin gel			
	Entrapment efficiency (%)		Vesicle size (nm)		Viscosity		Drug content	
	4-8 $^\circ\text{C}$	RT	4-8 $^\circ\text{C}$	RT	4-8 $^\circ\text{C}$	RT	4-8 $^\circ\text{C}$	RT
0	52.43	52.26	348.6	348.6	34360 $\pm$ 2.54	34360 $\pm$ 2.54	91.4	91.3
15	51.24	49.45	350.8	352.4	38640 $\pm$ 1.92	39530 $\pm$ 1.92	90.8	90.6
30	49.18	46.68	352.5	360.9	39640 $\pm$ 1.80	45440 $\pm$ 2.80	87.7	88.5

allowed a day rest. Then, UV irradiations were directed daily for 30 seconds using eight Philips TL12/20 W fluorescent sunlamp tubes which were positioned at 35 cm. The lamp emitted radiations between 210 nm-300 nm. The daily dose was kept at 99 mJ/cm<sup>2</sup>. Mice were kept unrestrained during the procedure. After the pigmentation was developed, the standard sample was applied. Four groups of three mice, and each were smeared twice daily with hydrogel (control), 2% Quercetin cream (standard), 2% ASP Quercetin gel. The applications continued for four weeks and the skin-whitening effect was measured on the skin every day and photographic images were taken.

#### *Skin irritation studies*

The albino rats of either sex weighing 20-22 g were used. Intact skin after hair removal before three days of experiments was taken for the study. Four groups of animals were taken and aspasomal gel was applied on test animal. A piece of cotton wool, soaked in saturated drug solution was placed on the back of mice taken as control. The animals were treated daily for seven days and the treated skin was visually examined for erythema and edema.

#### *Stability studies*

The optimized ASP dispersion batch ASP1 and its optimized batch of Aspasomal gel APG1 were kept in sealed vials (30 ml capacity) and stored at 2°-8°C (refrigerated temperature), 25 ± 2°C (room temperature, RT) for three months. Samples at each temperature conditions were taken at different time intervals and then observed under microscope for change in consistency (crystal formation) and drug retention. The drug retention was determined by centrifuging the sample at 12,000 rpm for 30 minutes and the supernatant analyzed for free drug. The experiments were performed in triplicate ( $n=3$ ).

### Results and Discussions

Photomicrography of aspasomes was done and it was found that multilaminar vesicles (Fig 1) are formed. The mean diameter of the aspasomal dispersions was 520 nm with a size distribution of 350 nm to 800 nm before the process of lyophilization. The entrapment efficiency of quercetin aspasomes was found to be maximum in (52.41%) in which the concentration of ascorbyl palmitate and cholesterol are equal. The Scanning Electron Microscopy analysis (SEM) of quercetin aspasomes is shown in Figure 1. It indicates that the aspasomes are bi-layered with almost spherical shape and uniform size. Zeta potential was determined and found to be between -27.6±3.8 to -48.2±2.4 and 0.433 to 0.970 respectively.

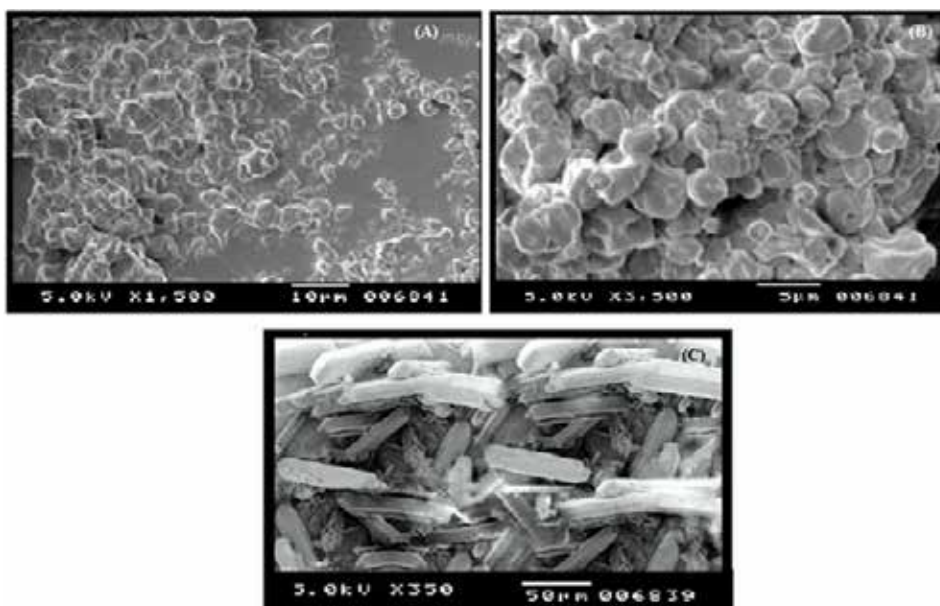


Figure 1: SEM image of optimized formulations

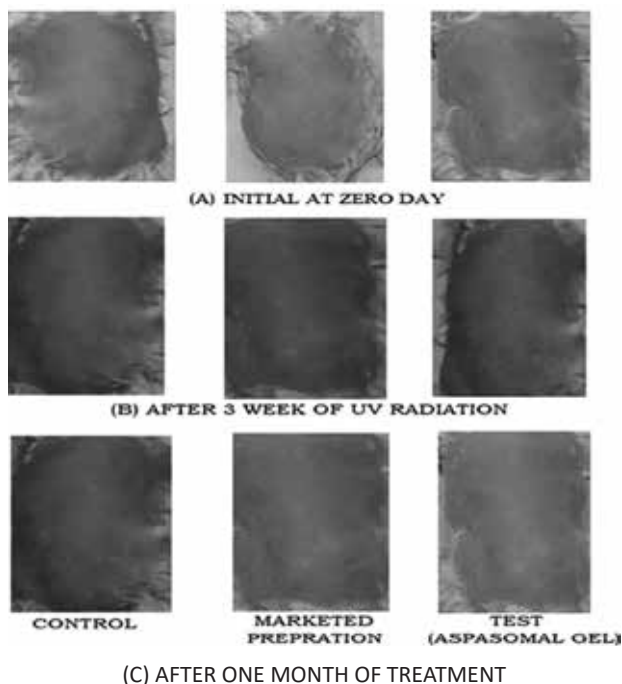


Figure 2: Depigmentation Activity of formulations

### *Aspasomal Gel Formulation*

Psychorheological characteristic of Aspasomal Gel was found that all the formulations were smooth, clear, transparent and shows no clogging which indicates the good texture of formulation and excellent homogeneity. In APG1, formulation concentration of carbopol 940 was 0.5 % so the viscosity of this formulation was found to be  $34,360 \pm 2.54$  cps which was gradually increased due to increase concentration of carbopol 940. The spreadability values are shown in range of 6.67 - 30.34 g.cm/s. However the spreadability values decreased with the concentration of carbopol in gel. The decrease in spreadability was due to the increase in the viscosity of gel. Extrudability of all gel formulation was excellent, the gel containing 0.5 carbopol extrudes easily and the value decreases with the concentration of carbopol 940 but all formulation shows very good to excellent extrudability. The pH of all formulations were in the range of 6-7 that suited the skin indicating the skin compatibility and it is primary requirement. There was no significant difference observed in the percentage of drug at various locations, indicating that the method used to disperse the aspasomal dispersion in the gel base is satisfactory. It was clear from the results that APG1

formulation was better than other formulation therefore it was taken for further study.

*In vitro* studies of developed system and aspasome gel was performed and in the study up to 78% of drug released rapidly and then the release was linear, after which the drug release rate decreased. In comparative percentage *in-vitro* drug release of Plain QZT, Aspasomal QZT, and Aspasomal QZT gel, it was found that the Aspasomal QZT-gel showed much slower release rate than Aspasomal QZT and plain QZT. In the drug permeation study, quercetin aspasomes permeation on topical application was better than the plain quercetin and aspasomal quercetin gel.

*In-vivo* skin irritation which was a visual examination for erythema and edema showed no evidence on test subjects during the two week treatment period. In the depigmentation studies, topical application of quercetin to hairless mice skin showed time related depigmentation of treated sites during the 30 day treatment (Fig 2). Topical application of the aspasomal gel did not cause any visible pigmentary change or local irritation. Definite visual depigmentation was noted after 15, 20, and 25 days, respectively. The visual depigmentation with marketed preparation (standard) and aspasomal QZT gel was observed.

Stability studies of ASP-QZT dispersion, ASP-QZT lyophilized and ASP-QZT gel was performed and after three months of storage at 4°C and RT, no significant changes in the size of all formulations were found. Though, the entrapment of the ASP formulations fell by about 5% in ASP-QZT dispersion. This result may be due to the crystal transition of cholesterol caused by temperature and storing. ASP-QZT lyophilized and ASP-QZT gel exhibited a good stability. No obvious changes in clarity of preparation were found.

Ascorbyl palmitate forms vesicles (Aspasomes) in presence of cholesterol, encapsulating Quercetin solution. The antioxidant potency and its capability to suppress pigmentation of the skin can be used for skin whitening and may find applications as drug delivery system for whitening or brightening the pigmented skin. Aspasomes also significantly

enhanced the transdermal permeation of Quercetin compound. The antioxidant property and lipophilic character suggest its use for transdermal drug delivery system.

### Conclusions

Ascorbyl palmitate is widely used in cosmetic and drug industry as an antioxidant. But the major problem that is associated with the chemical is its poor solubility and oxidative instability which is mostly seen in colloidal systems. When formulated as aspasomes it was found that the stability and penetration was better and this lead to enhanced bioavailability of the drug. The results indicate that aspasomal formulations are efficient in treatment of skin pigmentation disorders and produce whitening effect on skin. Thus, these can be used for antioxidant and skin whitening effects. Further studies are needed to follow the long term effects of the formulation.

### Conflicts of interest

The authors confirm no conflict of interest.

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