A Comparison of Explanation Methods of Encapsulation Efficacy of Hydroquinone in a Liposomal System

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ABSTRACT

One of the most important parameters describing the liposomal formulation of hydroquinone is encapsulation efficacy. For the efficacy evaluation of hydroquinone trapped in liposomal structure, there is a need to first separate liposome from the matrix surrounding it. There are various separation techniques; however, in this study, the three techniques of centrifuges with and without washing and dialysis were used. From among the laboratory techniques, an appropriate method is the one that offers responses with a high repeatability. The statistical calculations revealed that encapsulation efficacy with a direct method resulted from a separation via the techniques of dialysis and centrifuge without washing had the highest dispersion with SDs of 6.1 and 8.7, respectively, while the SD value in the technique of centrifuge with washing was 5.2. Through an indirect method, hydroquinone encapsulation efficacy showed the best repeatability with SD values of 2.8 and 2.1 by using the two techniques of centrifuge and centrifuge filtration, respectively. It seems that the treatments leading to the dilution of hydroquinone formulation would result in hydroquinone leakage and a reduction of encapsulation efficacy. It seems that measurement of hydroquinone encapsulation efficacy with an indirect method is a better choice; therefore, a centrifuge technique was utilized to report the mentioned efficacy at a speed of 45000 rcf and duration of 30 min due to having a reasonable price and ease of access.

Key Words: Hydroquinone; Liposome; Seperation Method; Centrifuge

INTRODUCTION

In recent years, drug carriers were developed to enhance the treatment efficacy of drugs by different mechanism including suspend release, thereby increasing the accumulation of drugs in target tissue and decreasing side effects [1-3]. Liposome has a spherical structure, composed of phospholipid in one or several layers [2] that can carry small to large [4] or hydrophobic to hydrophilic molecules [5]. Liposomes can cross skin barrier and deliver drug to deep layers of skin [6]. Some hyperpigmentation disorders such as skin melasma, freckles and lentigines are treated by using depigmenting agents containing hydroquinone [7-8]. Hydroquinone has diphenolic structure (1,4-dihydroxybenz en) and is a small (Mr 110.11 Da) molecule [9] that is used as a photographic developer [10], an intermediate for rubber processing chemicals in the production of mono and dialkyl ethers [11] and inhibitory of tyrosinase in the production of melanin [12]. Liposomal formulations are characterized by size, surface charge, number of layers and encapsulation efficacy. When preparing liposomal formulation, the molecules of drug that aren't entrapped inside the liposome are named free drug. The first step for

calculating encapsulation efficacy of liposomal formulation is the separation of free drug from liposomes containing drug. Method usded for separation should be fast and simple, and excessive sample dilution and the loss of drug from inside the liposome should be avoided [13]. In this study, several separation methods were applied to calculate the encapsulation efficacy of hydroquinone liposomal formulation that was used to treat hyperpigmentation disorders.

Material and Methods

Phospholipid S 100 (Phosphatidylcholine (PC) from soybean lecithin) was purchased from Lipoid Company. Cholesterol, hydroquinone, sodium metabisulfite, chloroform, and mono sodium dihydrogen phosphate (analytical grade) from Merck were used. Alpha ketopherol acetate and methanol from Applichem and Chemical Pars (analytical grade) were obtained respectively.

Instrumentation

Cecil 9500 Double Beam Spectrophotometer was used in spectrophotometric experiments. For the preparation of liposomal formulation, removal of organic solvents was carried out using Buchi rotary evaporator (R-210) and liposomal solution was homogenized by IKA T10 homogenizer. All the substances were weighed by Shimadzu balance with 0.1 mg accuracy. Dialysis tubing (cut off 12000 Da), centrifuge tube filter (cut off 10000 Da) and membrane filter paper (1000 and 800 nm) were obtained from Millipore. Centrifuge Sigma 3-K30 from Germany was used. Hemogene liposomal formulation was provided by extruder QF4 Swagelok from Canada. Malvern (nano-ZS) Particle size analyzer that was used to measure liposomal sample size was made in UK.

Preparation of HQ liposomes

Liposome dispersion samples were prepared using chloroform film method [14] with homogenization. Liposome formulations composed of S100 (7.78%), cholesterol (1.5%), alpha ketopherol (0.17%) and HQ (0.5%) w/v w dissolved in 15 ml chloroform and 5 ml methanol. Thin film layer was formed by vacuum-desiccation; the solution was then flushed with nitrogen gas for 1 min. The thin film was re-suspended in solution of 0.01M phosphate buffer (pH 6) with sodium metabisufite (0.1%) slowly, and swelled by shaking hand, vortex and final liposome solution was homogenized for 5 min by 2000 rpm speed of homogenizer. The liposomal particle size was reduced by sequential extrusion of large multilamellar vesicles through 1 and 0.8 µm of polycarbonate membranes.

Measurement of size

Liposomal sample was diluted 50 times by 0.01M phosphate buffer (pH 6) and liposomal particle size was measured by particle size analyzer.

Measurement of hydroquinone in liposomal matrix [15]

After separating the free drug from hydroquinone liposomal formulation, methanol was added to the sample and the intensity of absorption in 290 nm wavelength was evaluated by spectrophotometer.

Encapsulation Efficacy

Two methods, direct and indirect were used to calculate the encapsulation efficacy. After applying the separation methods using the direct method, pellet containing liposome particles were dissolved by methanol and hydroquinone entrapped to the liposome was measured; but in the indirect method, hydroquinone molecules in supernatant were measured and its result was reduced from the hydroquinone concentration in formulation. In this study, the concentration of hydroquinone in formulation was also evaluated.

Direct method

$$\frac{\text{drug Concentration in pellet}}{\text{drug concentrion in formularion}} * \%100$$

Indirect method

$$\frac{\text{drug concentration in formulation} - \text{drug concentration in supernatant}}{\text{drug concentration in formulation}} * \%100$$

Separation methods

a Centrifuge with washing (direct method): 2 ml samples were diluted by 1, 2, 4 and 8 times using buffer phosphate and was centrifuged at 45000 rcf for 30 min followed by 2 times washing with buffer, and

centrifugation for 30 min after each washing. Obtained pellet at the bottom of the tube was used to calculate encapsulation efficacy using direct method.

b.Centrifuge without washing (direct method): 2 ml samples were diluted by 1, 2, 4 and 8 times using buffer phosphate and was centrifuged at 45000 rcf for 30 min in 3 cycles, the encapsulation efficacy was calculated by measuring drug in pellet using direct method.

c.Centrifuge (indirect method): The diluted samples were centrifuged at 45000 rcf for 30 min in 3 cycles, then the supernatants and pellet were separated and the concentration of hydroquinone was measured in the supernatant.

***d. Dialysis*:** Dialysis tube containing 10 ml hydroquinone liposomal formulation was placed in a covered beaker for 12 h in mono sodium dihydrogen phosphate buffer (pH 5 and 0.005 M). This buffer was replaced by mono sodium dihydrogen phosphate buffer (pH 5 and 0.001M) for 6 h and then by mono sodium dihydrogen phosphate buffer (pH 5 and 0.0005M) to complete the dialysis for 24 h. The concentration of hydroquinone was measured in dialysis tube.

e.Centrifuge filtration: 5 ml liposomal hydroquinone was placed in the sample reservoir of centrifuge filtration tube and the unit was centrifuged at 10000 rpm for 30 min. Liposome remained at the top of the tube membrane and the free drug associated with supernatant was separated in the bottom filter cup. The concentration of hydroquinone in supernatant was measured.

SD calculation:

To calculate SD, liposomal samples with a dilution of 1 and 6 iterations were used.

RESULTS

Size measurement

Liposomes were extruded through millipore polycarbonate membranes of 1 and 0.8 µm pore size resulting in an average particle size of 800 nm, as measured by dynamic light scattering. The particle size distribution of the liposome achieved at 0.28 was acceptable.

Table 1.results of centrifuge with washing (direct method)

dilution	Hydroquinone in pellet(mg/ml)	Multiple in dilution reverse (mg/ml)	Encapsulation
1	1.96	1.96	%39
2	0.82	1.64	%33
4	0.31	1.24	%25
8	0.11	0.88	%18

Table 2. results of centrifuge without washing (direct method)

dilution	Hydroquinone in pellet(mg/ml)	Multiple in dilution reverse (mg/ml)	Encapsulation	Hydroquinone in supernatant (mg/ml)	total (mg/ml)
1	3.47	3.47	%71	2.49	5.96
2	1.46	2.92	%60	2.7	5.62
4	0.65	2.6	%53	2.95	5.55
8	0.18	1.44	%29	3.2	4.64

Table 3.results of centrifuge (indirect method)

dilution	Hydroquinone in supernatant (mg/ml)	Multiple in dilution reverse (mg/ml)	Hydroquinone in formulation(4.84mg/ml) - supernatant	Encapsulation
1	2.49	2.49	2.35	%48
2	1.135	2.7	2.14	%44
4	0.73	2.95	1.88	%39
8	0.41	3.2	1.54	%32

Table 4. comparative of results in various methods

Centrifuge filtration	47%
Centrifuge with washing (direct)	39%
Centrifuge without washing (direct)	71%
Centrifuge (indirect)	48%
Dialysis	7%

Table 5. standard deviation of various technics

Type of measurement method of encapsulation efficacy	Technique	SD
Direct	**Centrifuge with washing**	5.2
	Dialysis	6.1
	Centrifuge without washing	8.7
Indirect	**Centrifuge filtration**	2.1
	Centrifuge	2.8

Centrifuge filtration is one of the methods used to separate liposomal formulations and is capable of separating liposomal particles from a formulation solution. Formulations are diluted so that the pores of the membrane of a centrifree unit may not be blocked by liposomal particles before the entire formulation passes [14]. In this study, the effect of dilution on the evaluation of encapsulation efficacy was investigated using several separation techniques. Liposomal formulations were diluted at 1, 2, 4 and 8 ratios, and the separation operation was performed by the centrifuge technique through the direct method through washing, without washing, and the direct method, whose results are shown in Tables 1, 2 and 3. The three tables show that, as the formulation becomes more dilute, the encapsulation efficacy decreases. It seems that dilution causes the medicine to leak from the liposomal structure, thus dilution is not appropriate when evaluating liposomal formulations. In dialysis studies, where the environment around the dialysis bag is very dilute, an encapsulation efficacy of 7% was obtained. Some researchers have shown that dialysis causes the leakage of small non-electrolyte molecules [16-17]. Hence, the process of dilution or washing causes the leakage of hydroquinone. In the techniques that dilute liposomal formulation, hydroquinone leakage from liposomes will increase the dispersion index of responses, thereby reducing the parameter of repeatability. Therefore, In order to perform a lesser dilution during the separation operation, the centrifuge technique was used through the direct method and without washing. To assess this method, the dosage of the medicine, both in the pellet and supernatant, was measured, and the sum of these amounts is expected to equal the total medicine in the formulation. It is evident from Table 2, the encapsulation efficacy is higher in this method than in the method combined with washing, and the total medicine in the pellet and supernatant is greater than the whole formulation. Also, from among the techniques employed, this method demonstrates the highest SD (table 5). It can be said that it was the pellet associated with some supernatant that caused a false increase in the encapsulation efficacy. Altogether, the direct method cannot be used to measure the encapsulation efficacy of hydroquinone. Hence, indirect methods were used. Non-encapsulated hydroquinone was solved uniformly in the space of the supernatant; therefore the encapsulation efficacy can be obtained by measuring the free hydroquinone and reducing the total amount of hydroquinone. In this study, a centrifuge was used to separate the transparent supernatant solution from the pellet. The amount of medicine in the supernatant was calculated as 2.49 mg/ml and was 4.84 mg/ml within the whole formulation, while the encapsulation efficacy was found to be 48%.

The indirect method, with the aid of the centrifuge filtration technique was used to calculate the encapsulation efficacy. This time, after a slight collection of a transparent supernatant in a filtrate-vial, the amount of free hydroquinone was calculated as 2.53 mg/ml, and based on the total amount of hydroquinone (4.84mg/ml), the encapsulation efficacy was calculated as 47%. As can be seen, the results of the indirect method in the two techniques of centrifuge and centrifuge filtration technique are almost the same, and repeatability of responses (SD 2.8 and 2.1) is higher than those of the other methods. Since employing a simple and inexpensive method is one of the objectives of designing this test, the centrifuge technique through the indirect method was used to evaluate the encapsulation efficacy of hydroquinone in the liposomal formulation. In the process of optimizing the conditions of the centrifuge, different speeds and times were used, and eventually, due to the large size of liposomal particles (800 nm), a speed of 45000 rcf and time of 30 min were obtained as the best separation condition.

DISCUSSION

To select a technique for separating liposomal formulations, attention should be paid to the formulation characteristics such as the size of particles and medicine. Each experimental trend with great effects on the assessment of hydroquinone encapsulation efficacy would lead to an increased range of SD, thus affecting the test repeatability. Those techniques are acceptable that have a high reproducibility. Any substance or formulation may be more sensitive to some treatments; yet, the best experimental method can be achieved by using several different techniques. During laboratory processes, small molecules of hydroquinone (110 Da) with a distribution coefficient of 0.54, are highly probable to leak from liposomes [18-19]. Hence, some separation processes, such as dilution or washing, and techniques such as dialysis are not suitable for separating them since the scattering replies increases. It was also observed in this study that the total amount of hydroquinone decreased after preparing the formulation; thus, it is necessary to measure the total amount of medicine in order to report the encapsulation efficacy. Altogether, measuring the encapsulation efficacy of hydroquinone which is a small molecule and soluble in water was made possible through an available technique, such as the centrifuge technique through the indirect method.

"The authors declare no conflict of interest"

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