An approach for determining the accelerated stability during the optimization of ellagic acid loaded transfersomes using the computer program

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Abstract

Introduction: In the development of pharmaceutical products, the objective of the formulation optimization is to design and develop the proper dosage form that results in higher efficacy, safety and stability. The formulation efficacy and safety can be assessed during the optimization process. The evaluations for the formulation efficacy and safety saved time more than the formulation stability study. The stability evaluation takes a lot of time for the long term stability study, so several researchers have chosen the accelerated stability instead. However, at least 2-3 months were spent for the accelerated stability evaluation. The objective of this study was to minimize the time and measurement samples for determining the accelerated stability during the optimization of ellagic acid loaded transfersomes using the computer program. **Methods:** Ten model formulations of transfersomes consisting of a constant percentage of phosphatidylcholine, ellagic acid and various molar percentages of cholesterol and oleic acid from 0-90%mol. The transfersome formulations were experimentally prepared and investigated. To evaluate the stability, all transfersome formulations were kept in the stability chamber at 45^{O}C for 12 h and the refrigerator at 4^{O}C for 12 h, for3 cycles. The sample was collected and measured through the physicochemical characteristics, e.g. vesicle size, polydispersity index (PDI), surface charge and entrapment efficiency. The Design Expert® was applied for determining the accelerated stability during the formulation optimization. **Results:** The results suggested that the computer program was a powerful methodfor estimating the stability of the formulation during the optimization process. Moreover, the Design Expert® obviously showed the relationship between the formulation components and the formulation stability. **Conclusion:** The utilization of the computer program in this study was succeeded in showing the feasibility of the approach for determining the accelerated stability during the formulation optimization with the minimization of time and measurement samples.

Keywords: Transfersomes, Stability evaluation, Accelerated stability, Optimization

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วิธีการศึกษาความคงตัวในสภาวะเร่งในระหว่างการหาสูตรตำรับที่เหมาะสมที่สุดของทรานสเฟอร์โซมกักเก็บกรดเอลลาจิกโดยใช้โปรแกรมคอมพิวเตอร์

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บทคัดย่อ

บทนำ : ในการพัฒนาเภสัชภัณฑ์ วัตถุประสงค์ของการหาสูตรตำรับที่เหมาะสมคือการออกแบบและพัฒนาสูตรตำรับที่มีความเหมาะสมทั้งด้านประสิทธิภาพ ความปลอดภัย และความคงตัวสูง ประสิทธิภาพและความปลอดภัยของสูตรตำรับสามารถทดสอบในระหว่างกระบวนการหาสูตรตำรับที่เหมาะสม ระยะเวลาที่ใช้ในการประเมินประสิทธิภาพและความปลอดภัยของสูตรตำรับประหยัดเวลากว่าการศึกษาความคงตัวของสูตรตำรับ การประเมินความคงตัวใช้เวลานานในการศึกษาความคงตัวในระยะยาว ดังนั้นนักวิจัยหลายคนจึงเลือกวิธีศึกษาความคงตัวในสภาวะเร่งแทน อย่างไรก็ตาม การประเมินความคงตัวในสภาวะเร่งใช้เวลาอย่างน้อย 2-3 เดือน วัตถุประสงค์ของการศึกษานี้เพื่อลดระยะเวลาและจำนวนตัวอย่างที่ทดสอบสำหรับการหาความคงตัวในสภาวะเร่งในระหว่างการหาสูตรตำรับที่เหมาะสมที่สุดของทรานสเฟอร์โซมกักเก็บกรดเอลลาจิกโดยใช้โปรแกรมคอมพิวเตอร์ **วิธีดำเนินการวิจัย:** สูตรตำรับต้นแบบ 10 สูตร ประกอบด้วยฟอสฟาทิดิลโคลีนและกรดเอลลาจิกในปริมาณร้อยละคงที่ และแปรผันร้อยละโมลาร์ของคอเลสเตอรอลและกรดโอเลอิกจากร้อยละ 0-90 สูตรตำรับทรานสเฟอร์โซมถูกเตรียมขึ้นและประเมินคุณลักษณะทางเคมีกายภาพและความคงตัว สูตรตำรับทรานสเฟอร์โซมทุกสูตรถูกเก็บในตู้ศึกษาความคงตัว 45 องศาเซลเซียส เป็นเวลา 12 ชั่วโมง และในตู้เย็น 4 องศาเซลเซียส เป็นเวลา 12 ชั่วโมง จำนวน 3 รอบ ตัวอย่างจะถูกสุ่มนำไปวัดคุณลักษณะทางเคมีกายภาพ คือ ขนาดอนุภาค ดัชนีการกระจายขนาด ประจุที่พื้นผิว และการกักเก็บตัวยา จากนั้นจะประยุกต์ใช้โปรแกรม Design Expert® ในการหาความคงตัวในสภาวะเร่งระหว่างการหาสูตรตำรับที่เหมาะสมที่สุด **ผลการวิจัย:** พบว่าโปรแกรมคอมพิวเตอร์เป็นวิธีที่มีประสิทธิภาพในการทำนายความคงตัวของสูตรตำรับในระหว่างการหาสูตรที่เหมาะสมที่สุด นอกจากนี้ Design Expert® แสดงความสัมพันธ์ระหว่างส่วนประกอบและความคงตัวของสูตรตำรับอย่างชัดเจน **สรุปผลการวิจัย:** การใช้โปรแกรมคอมพิวเตอร์ในการศึกษานี้ประสบความสำเร็จในการแสดงให้เห็นถึงความเป็นไปได้ในการหาวิธีการศึกษาความคงตัวในสภาวะเร่งในระหว่างการหาสูตรตำรับที่เหมาะสมที่สุดที่ลดระยะเวลาและจำนวนตัวอย่างที่ทดสอบ

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Introduction

Previously, the development of various pharmaceutical dosage forms has been based on the trial and error method to obtain an acceptable formulation with multiple desirable characteristics including high efficacy, high safety and high stability. The relationships between formulation compositions and their physicochemical characteristics were not fully understood (Duangjit, 2012). A high concentration of some pharmaceutical excipient may relate to the improvement of the efficacy of the formulation for instance the incorporation of surfactant as a penetration enhancer can enhance the skin permeability of topical formulation e.g., liposomes, microemulsions, micelles, solid lipid nanoparticles (Williams and Barry, 2004). On the other hand, the high percentage of surfactant used in the formulation resulted in decreasing the formulation stability of some dosage forms including liposome formulation (Briuglia, 2015). Moreover, skin irritation (as the formulation safety) of high percentage of surfactant should be concurrently considered at the same time. Thus, the design and development of pharmaceutical products and manufacturing processes should ensure a predefined quality by understanding of how the factors influenced the quality of pharmaceutical products (Kikuchi and Takayama, 2010). The detail of the concept of quality by design (QbD) has been prescribed in the ICH Q8 guideline (Yu, 2008).

In recent years, the computational method was applied to obtain an acceptable formulation for multi-appropriate formulation with high efficacy, stability and safety (Duangjit, 2014; Nishikawa, 2008; Nishikawa, 2011; Yu, 2008). The advantage of the computational method that overcomes the conventional comparative method (the trial and error method) was to understand the complicated relationships between various formulation factors (causal factors; X_n) and their formulation characteristics (response variable; Y_n). Moreover, the number of measurement samples needed for estimating by the computer program was fewer compared to the conventional comparative method. Thus, the computational method was helpful to reduce the time and cost during the formulation development process.

In the optimization of liposomes for transdermal drug delivery, the previous study showed that the physicochemical characteristics values of the optimal formulation (e.g., vesicle size, size distribution, zeta potential, entrapment efficiency and skin permeation flux) obtained from the computer program were agreed well with the experimental values (Duangjit, 2012). Generally, the objective of the formulation optimization is to design and develop the proper dosage form that results in higher efficacy, safety and formulation stability. The formulation efficacy and safety can be assessed during the optimization process. The evaluations for the formulation efficacy and safety saved time more than the formulation stability study. The computer program can used to optimize the optimal condition for both formulation efficacy and safety simultaneously. However, the stability evaluation takes a lot of time for the long term stability study. The optimal condition for the formulation stability is still complicated and challenging for several researchers. The

alternative choice of the formulation stability study was the accelerated stability. However, at least 2-3 months were spent for the accelerated stability evaluation. The determination of the formulation stability is still required for every effective pharmaceutical product. The limitation of formulation stability investigation was not only a long time consuming, but also the large number of measurement samples are needed. To overcome this problem, the objective of this study was to minimize the time and measurement samples for determining the accelerated stability during the optimization of ellagic acid loaded transfersomes using the computer program.

Materials and methods

Materials

Phosphatidylcholine (PC) was a special gift obtained from LIPOID GmbH (Cologne, Germany). Cholesterol (CH) was supplied from Carlo Erba Reagenti, (Strada Rivoltana, Italy). Oleic acid (OA) was purchased from Sigma Aldrich® (MO, USA). Ellagic acid (EA) supplied from Acros Oranics (New Jersey, USA). All chemicals used in this study were analytical grades.

Experimental design

A two factor spherical second order composite experimental design was used for determining the accelerated stability during optimizationin this study (Duangjit, 2012). Two components in the EA loaded transfersomes, including the membrane stabilizer (X_1) and the penetration enhance (X_2) were selected as formulation factors. The components in the EA loaded transfersomes were studied by varying their concentration simultaneously. The upper and lower limits of the levels of each component were experimentally demonstrated and set as follows equation:

$$10 < X_1 < 40\ (\%) \quad (1)$$

$$10 < X_2 < 40\ (\%) \quad (2)$$

Preparation of EA loaded transfersomes

Ten model formulations of transfersome were obtained from the two factor spherical second order composite experimental design (Duangjit, 2012) as shown in Table 1. Transfersomes containing a controlled amount of phosphatidylcholine (PC), ellagic acid (EA) and various percentages of cholesterol (CH) and oleic acid (OA) were prepared by the sonication method. The PC, EA, CH and OA were each briefly dissolved in chloroform : methanol (2:1 v/v). In preparing EA loaded transfersomes, the materials were deposited in a test tube, and the solvent was evaporated with nitrogen gas. The lipid film was placed in a desiccator at least 6 h to remove the remaining organic solvents. The dried lipid film was rehydrated with phosphate buffer solution pH 7.4. Following hydration, the dispersion was sonicated in a bath for 30 min and then probe-sonicated for 10 min.

Table 1 The composition of ellagic acid loaded transfersome formulation

Form	Concentration (mM)			
	PC	Chol	OA	EA
1	10	14.4	14.4	0.1
2	10	35.6	35.6	0.1
3	10	35.6	14.4	0.1
4	10	10.0	35.6	0.1
5	10	40.0	25.0	0.1
6	10	25.0	25.0	0.1
7	10	25.0	10.0	0.1
8	10	25.0	40.0	0.1
9	10	25.0	25.0	0.1
10	10	25.0	25.0	0.1

Characterization of EA loaded transfersome

The vesicle size, the polydispersity index and the surface charge of the EA loaded transfersome were determined by a Zetasizer (Malvern Instrument, UK). All measurement samples were performed at 25°C. Twenty µL of the transfersomes were diluted with 1480 µL of deionized water. At least three independent samples were taken, and the vesicle size, the polydispersity index and the surface charge were measured at least three times.

Determination of entrapment efficiency of EA loaded transfersome

The concentration of EA in the transfersome formulation was determined by HPLC analysis after disruption of the transfersomes with methanol at a 1:1 volume ratio and appropriate dilution with phosphate buffer solution pH 7.4. The transfersome/methanol mixture was centrifuged at 10,000 rpm for 10 min. The supernatant was filtered with a 0.45 µm nylon syringe filter. The entrapment efficiency of the EA loaded transfersomes was calculated by Eq. 3. Where C_L is the concentration of EA loaded in transfersome as described in the above methods and C_i is the initial concentration of EA added into the formulation.

% entrapment efficiency = $(C_L/C_i) \times 100$(3)

Stability evaluation

The EA loaded transfersome formulations were kept in glass bottles with plastic plugs and stored at 45±1°C for 12 h and then moved to 4±1°C for 12 h, for 3 cycles (totally 3 days). The physicochemical stability of the EA loaded transfersome formulations, such as the vesicle size, the polydispersity index and the surface charge were evaluated by Zetasizer. The EA remaining in the formulation was determined by HPLC at cycles 1, 2 and 3. The results of the physicochemical characterization immediately after preparation (at initial) were used as a control, and EA entrapped in the formulation at the initial was also normalized to 100%.

HPLC analysis

The HPLC (ThermoDionex UV/Vis, USA) was used to analyze the measurement samples. The analytical column was Hyper Clone 5u ODS C-18 column (250x4.6 mm), and the mobile phase consisting of acetronitrile/0.1% phosphoric acid under the gradient mode at 27°C, set at a flow rate of 0.8 mL/min, and the UV detector was set at 254 nm for all determinations. The calibration curve for

EA was in the range of 1-10 μg/mL with a correlation coefficient of 0.9992.

Computational stability estimation

The experimental design was studied based on two formulation compositions: CH (X_1) and OA (X_2). The physicochemical characteristics e.g., vesicles size (Y_1), polydispersity index (Y_2), surface charge (Y_3) and entrapment efficiency (Y_4) were taken as response variables. The two factor spherical second order composite experimental design was used to estimate the relationship between the formulation composition and the formulation stability. The response surfaces of all transfersome formulations were estimated and sketched by Design-Expert® Software (Stat-Ease, Inc. MN, USA). The best fitting mathematics (e.g. linear, quadratic, cubic and special cubic) was suggested based on the transfersome summary statistics: the standard deviation (SD), the multiple correlation coefficient (R^2), the adjusted multiple correlation coefficient (adjusted R^2), the predicted multiple correlation coefficient (predicted R^2) and the predicted residual sum of square (PRESS), were verified by Design-Expert® Software.

Results and discussion

Influence of formulation stability on the vesicle size

The influence of the formulation stability on the vesicle size is shown in Figure 1. The results indicated that the percentage of formulation components affected the vesicle size and also the formulation stability of the transfersome formulations. However, the results analyzed by the conventional comparative method were not clear enough to predict the influence of CH and OA on the vesicle size as shown in Figure 1 (A). The vesicle size of day 0 and cycle 1-3 was not significant difference in this study. The contour plots of the transfersome formulations estimated by the computational method are illustrated in Figure 1 (B) and (C). The percentage of CH and OA used in the transfersome formulation was represented in the x-axis and y-axis, respectively. The high value of vesicles size was defined as the red contour area while the blue contour area displayed the small value of vesicles size. The relationship between the formulation components and the formulation stability (as the vesicle size) was easily understood under the estimation using the computer program. The influence of CH and OA on the vesicle size was exhibited in Figure 1 (B). The difference in the shedding of contour plot among the day 0, cycle 1, cycle 2 and cycle 3 presented the trend of changing size after the incubation. Moreover, the computer program can be used for calculating the difference between the vesicle size at the initial formulation (day 0) and the formulation after the accelerated stability testing (cycle 1, cycle 2 and cycle 3) as shown in Figure 1 (C). The results suggested that the increase in the percentage of CH and OA resulted in the decrease in the vesicle size. The addition of CH resulted in the slight decrease in the vesicle size of transfersomes due to cholesterol causing the bilayer to be more compact (Verma, 2003) while the addition of OA resulted in the decrease in the vesicle size of transfersomes

because of its intrinsic properties that has a high radius of curvature (Elsayed, 2007). After the cycle 1 of incubation period, the transfersome formulations slightly decreased in the vesicle size. At the end of the accelerated stability testing (cycle 3), the vesicle size of the transfersome formulations was significantly decreased compared to the initial formulation (day 0). These could be summarized that the CH and OA significantly affected the vesicle size and the stability of transfersome formulations (Duangjit, 2011).

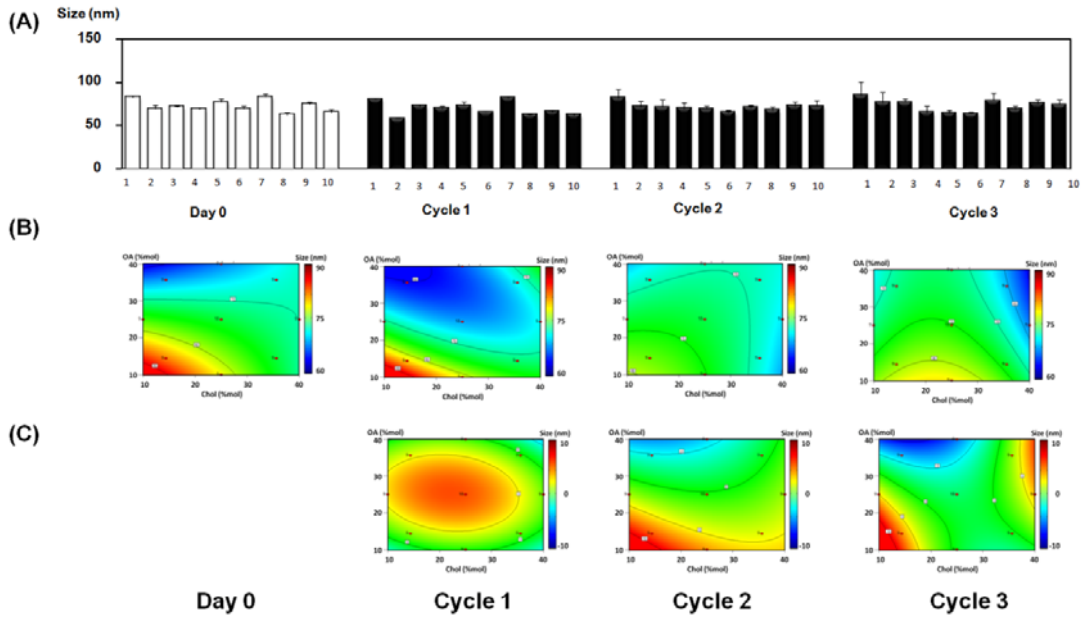

Figure 1 The determination of the vesicle size on the formulation stability (A) conventional comparative method, (B) contour plot of computational method and (C) contour plot of the different between Day 0 and cycle n

Effect of composition on the polydispersity index

The influence of the formulation stability on the polydispersity index of size distribution is shown in Figure 2. The stability data compared among day 0, cycle 1, cycle 2 and cycle 3 as shown in Figure 2 (A). The contour plots of the polydispersity index of the transfersome formulations estimated by the computational method are illustrated in Figure 2 (B) and (C). The results showed that the polydispersity index of the transfersome formulations was not affected by the percentage of formulation components (both CH and OA). However, the contour plots displayed more clearly than the conventional comparative method. The difference between the polydispersity index at the initial formulation (day 0) and the formulation after the accelerated stability testing (cycle 1, cycle 2 and cycle 3) as shown in Figure 2 (C) can be utilized for predicting the formulation stability of the transfersome formulations. Moreover, the method of preparation may affect the

polydispersity index of this system as confirmed by Yordanov (Yordanov, 2010).

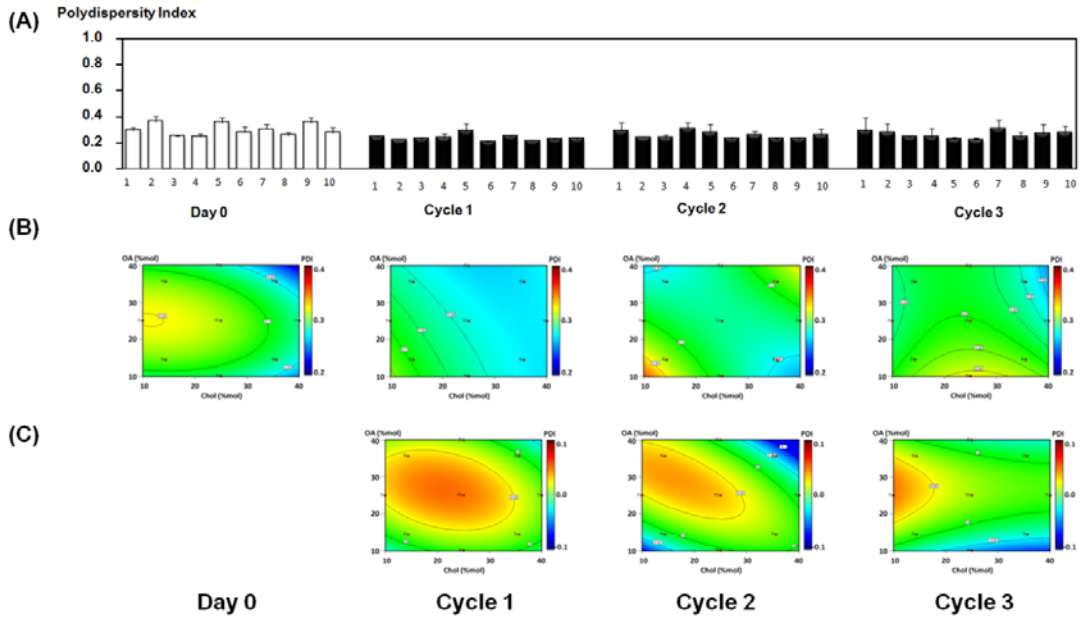

Figure 2 The determination of the polydispersity index on the formulation stability (A) conventional comparative method, (B) contour plot of computational method and (C) contour plot of the different between Day 0 and cycle n

Effect of composition on the surface charge

The effect of the formulation stability on the zeta potential or surface charge is shown in Figure 3. The results revealed that the percentage of formulation components affected the surface charge and the stability of the transfersome formulations. However, the conventional comparative method compared among day 0, cycle 1, cycle 2 and cycle 3 as shown in Figure 3 (A) is still complicated to understand. The contour plots of surface charge of the transfersome formulations as illustrated in Figure 3 (B) were easy to understand the conventional comparative method. The result indicated that CH did not affect the surface charge due to its intrinsic properties as the non-polar molecule (Junyaprasert, 2008). The surface charge of all transfersome formulations was negative; this result may be owing to the net charge of the formulation component (PC, CH, OA and EA). It has been reported that the increase in negative charges of membrane surface was due to the addition of fatty acids into the membrane (Doltchinkova and Nikolov, 1997 ; Vernotte, 1983). The difference between the surface charge at the initial formulation (day 0), cycle 1, cycle 2 and cycle 3 is shown in Figure 3 (C). The surface charge of transfersome formulation after cycle 1, cycle 2 and cycle 3 of the

accelerated stability testing was significantly different from the surface charge at day 0 formulation. The incorporation of CH up to 40%mol led to increasing the formulation stability of transfersomes, at cycle 2 and 3 as displayed in Figure 3 (C).

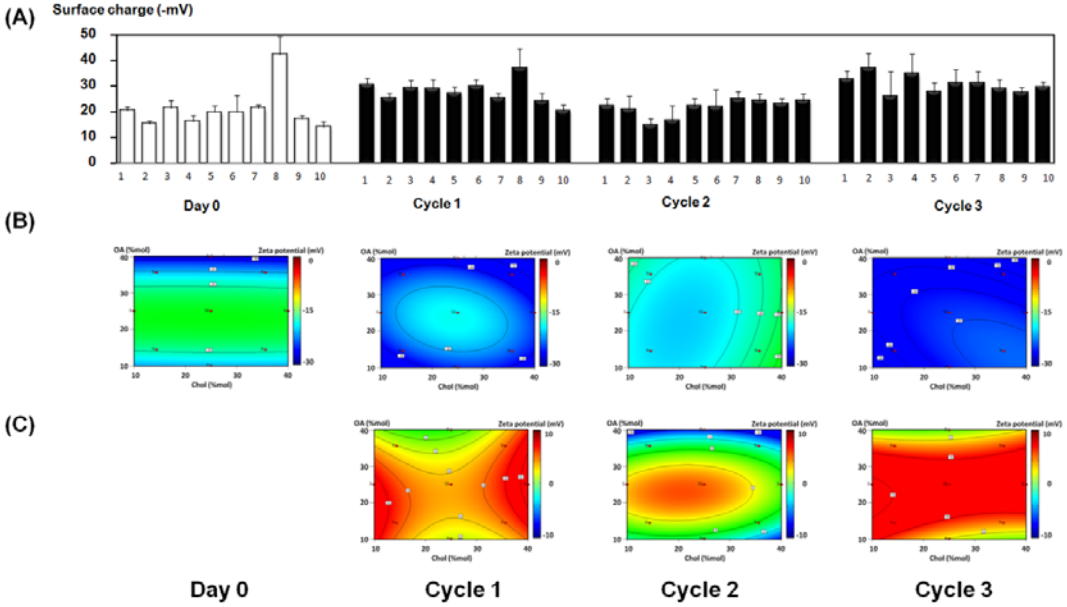

Figure 3 The determination of the surface charge on the formulation stability (A) conventional comparative method, (B) contour plot of computational method and (C) contour plot of the different between Day 0 and cycle n

Effects of composition on the entrapment efficiency

The consequence of the formulation stability on the entrapment efficiency is shown in Figure 4. The results indicated that the degradation of EA was independent of the formulation composition but dependent on the storage time and temperature. This result was in agreement well with the previous studies (Duangjit, 2011; Duangjit, 2014; Raffy and Teissié, 1999; Sulkowski, 2005). The entrapment efficiency of the transfersome formulations was significantly decreased after cycle 1 of the stability testing as shown in Figure 4 (B). The incorporation of CH resulted in the decrease in the degradation of EA while the addition of OA resulted in the increase in the degradation of EA at cycle 3 as shown in Figure 4 (C).

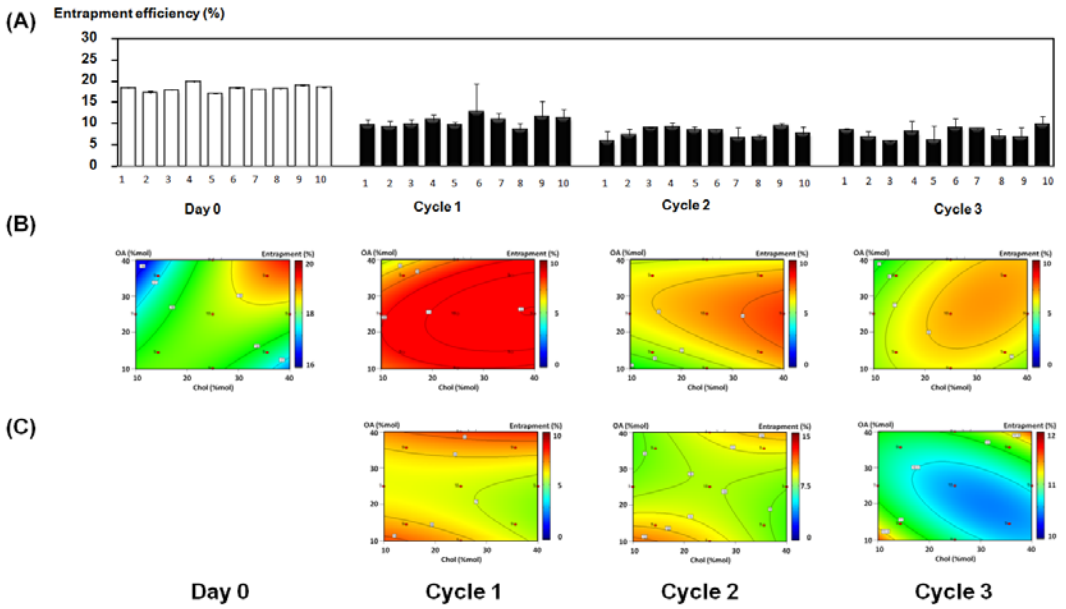

Figure 4 The determination of the entrapment efficiency on the formulation stability (A) conventional comparative method, (B) contour plot of computational method and (C) contour plot of the different between Day 0 and cycle n

Conclusion

The utilization of the computer program in this study was succeeded in showing the feasibility of the approach for determining the accelerated stability during the optimization with the minimization of time (totally 3 days) and measurement samples (at least 30 samples). This study revealed that the computation method was an attractive alternative method for analyzing the complicated data relationship between the formulation components and their stability. The further study requires evaluating the accuracy and reliability of the predicted value by the experiment.

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References

Briuglia ML, Rotella C, McFarlane A, Lamprou DA, Influence of cholesterol on liposome stability and on in vitro drug release. *Drug delivery and translational research* 2015; 5(3): 231-242.

Doltchinkova V, Nikolov R, Unsaturated fatty acids induced changes in surface charge density and light-scattering in pea thylakolid. *Bulg J Plant Physiol* 1997 23(1-2): 3-11.

Duangjit S, Mehr LM, Kumpugdee-Vollrath M, Ngawhirunpat T, Role of simplex lattice statistical design in the formulation and optimization of microemulsions for

transdermal delivery. *Biological & pharmaceutical bulletin* 2014; 37(12): 1948-1957.

Duangjit S, Obata Y, Sano H, Kikuchi S, Onuki Y, Opanasopit P, et al., Menthosomes, novel ultradeformable vesicles for transdermal drug delivery: optimization and characterization. *Biological & pharmaceutical bulletin* 2012; 35(10): 1720-1728.

Duangjit S, Opanasopit P, Rojanarata T, Ngawhirunpat T, Characterization and in vitro skin permeation of meloxicam-loaded liposomes versus transfersomes. *Journal of drug delivery* 2011; 2011: 418316.

Duangjit S, Opanasopit P, Rojarata T, Ngawhirunpat T, Physicochemical stability of meloxicam loaded vesicle formulation: effect of cholesterol. *IJPS* 2014; 9: 173.

Elsayed MM, Abdallah OY, Naggar VF, Khalafallah NM, Lipid vesicles for skin delivery of drugs: reviewing three decades of research. *International journal of pharmaceutics* 2007; 332(1-2): 1-16.

Junyaprasert VB, Teeranachaideekul V, Supaperm T, Effect of Charged and Non-ionic Membrane Additives on Physicochemical Properties and Stability of Niosomes. *AAPS PharmSciTech* 2008; 9(3): 851-859.

Kikuchi S, Takayama K, Multivariate statistical approach to optimizing sustained-release tablet formulations containing diltiazem hydrochloride as a model highly water-soluble drug. *International journal of pharmaceutics* 2010; 386(1-2): 149-155.

Nishikawa M, Onuki Y, Isowa K, Takayama K, Formulation optimization of an indomethacin-containing photocrosslinked polyacrylic acid hydrogel as an anti-inflammatory patch. *AAPS PharmSciTech* 2008; 9(3): 1038-1045.

Nishikawa M, Onuki Y, Okuno Y, Takayama K, Impact of the state of water on the dispersion stability of a skin cream formulation elucidated by magnetic resonance techniques. *Chemical & pharmaceutical bulletin* 2011; 59(3): 332-337.

Raffy S, Teissié J, Control of Lipid Membrane Stability by Cholesterol Content. *Biophys J* 1999; 76(4): 2072-2080.

Sulkowski WW, Pentak D, Nowak K, Sułkowska A, The influence of temperature, cholesterol content and pH on liposome stability. *J Mol Struct* 2005; 744–747: 737-747.

Verma DD, Verma S, Blume G, Fahr A, Particle size of liposomes influences dermal delivery of substances into skin. *International journal of pharmaceutics* 2003; 258(1-2): 141-151.

Vernotte C, Solis C, Moya I, Maison B, Briantais J-M, Arrio B, et al., Multiple effects of linolenic acid addition to pea thylakoids. *Biochim Biophys* 1983; 725(2): 376-383.

Williams AC, Barry BW, Penetration enhancers. *Adv Drug Deliv Rev* 2004; 56(5): 603-618.

Yordanov G, Bedzhova Z, Dushkin C, The effect of preparation method on the size distribution of poly(butylcyaoacrylaye) nanoperticles loaded with chlorambucil. *Nanosci Nanotechnol* 2010; 10: 162-165.

Yu LX, Pharmaceutical quality by design: product and process development, understanding, and control. *Pharmaceutical research* 2008; 25(4): 781-791.