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Optimization of the preparation of sonogenic phospholipids-based microbubbles by using central composite experimental design and response surface methodology

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Abstract: Sonogenic microbubble agent is a newly developed drug targeting delivery system, which uses ultrasonic beam to enhance the delivery of drug and gene to targeted cells and tissues. In this paper, the preparation of sonogenic phospholipids-based microbubbles was optimized by using central composite experimental design (CCD) and response surface methodology (RSM). Hydrogenated egg phosphatidylcholine (EPC), Tween 80 and polyethylene glycol 1500 (PEG 1500) were important components affecting the concentration of 2 - 8 μm microbubbles in the preparation. The combined effects of these three factors were analyzed by CCD and optimized by RSM. Evaluation variable was the concentration of 2 - 8 μm microbubbles. Overall desirability was fitted to a second-order polynomial equation, through which three dimensional response surface graphs were produced. Optimal experimental conditions were selected from the stationary point of the response surfaces. The stability of the sonogenic phospholipids-based microbubbles by the optimal formulation was investigated by accelerated experiment. The contrast effect *in vivo* of the optimal formulation was investigated. Foreign market product SonoVue ® was used as the control. From the results, all the three factors had positive effects on the concentration of 2 - 8 μm microbubbles. The optimal condition in the preparation of phospholipids-based microbubbles was obtained as following: EPC 8.35 mg, Tween 80 21.68 mg and PEG 1500 201 mg. The mean value of the concentration of 2 - 8 μm microbubbles in rechecking experiment reached $8.60 \times 10^9 \cdot mL^{-1}$. From the accelerated experiment, phospholipids-based microbubbles showed good physical stability. The intensity (relative unit) and duration of the contrast effect by the optimal formulation were 4.47 ± 0.15 and (302 ± 7) s respectively, which showed little difference with foreign market product SonoVue ® [4.28 ± 0.13, (309 ± 8) s]. The optimal formulation selected by CCD and RSM showed high microbubble concentration, good physical stability and effective sonogenic contrast effect.

Key words: sonogenic phospholipids-based microbubble; central composite design; response surface methodology; contrast effect

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星点设计结合效应面法优化声学脂质微泡的制备

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摘要: 声学微泡是近年发展起来的药物靶向递送系统,利用超声波作用促进药物或基因定位释放到细胞或组织中。本文利用星点设计(CCD)结合效应面法(RSM)优化声学脂质微泡制备条件。蛋黄磷脂、Tween 80 和聚乙二醇

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1500 是影响 2 ~ 8 μm 微泡浓度的主要因素。本文应用星点设计综合考察这些因素,利用效应面优化法得到最佳处方。实验评价指标为 2 ~ 8 μm 粒径的微泡浓度。采用多元二次方程对实验结果进行拟合,从而产生三维效应曲面图,最佳处方条件可从三维效应曲面的顶点得到。优化实验得到的最佳处方进行加速试验,考察稳定性。通过体内造影效果实验,研究本品的声学效应,并与国外上市产品 SonoVue® 进行对照。结果表明,3 个考察因素对 2 ~ 8 μm 微泡浓度均有影响,最佳处方配比为:蛋黄磷脂 8.35 mg, Tween 80 21.68 mg 和聚乙二醇 1500 201 mg。所制备的 2 ~ 8 μm 微泡浓度平均值达到 $8.60 \times 10^9 \cdot mL^{-1}$。加速试验结果显示脂质微泡物理稳定性良好。本品最佳处方体内造影强度(相对强度)和持续时间分别为 4.47 ± 0.15 和(302 ± 7)s,与国外上市产品 SonoVue® [4.28 ± 0.13 和(309 ± 8)s]无明显差异。星点设计结合效应面法筛选出的声学脂质微泡浓度高,物理稳定性和声学造影效果好。

关键词:声学脂质微泡;星点设计;效应面法;造影

Ultrasound contrast agent can overcome the fundamental limitations on the ability of ultrasound alone to differentiate between healthy and diseased tissues[1]. Most ultrasound contrast agents are composed of sonogenic microbubbles, which are filled with perfluorocarbons to control the bubble size and maintain sufficiently stable in circulation[2]. Among various agents, phospholipid-stabilized microbubbles, such as SonoVue®, are stable and resistant to pressure[3,4].

It has been an interesting issue of research to study ultrasonic-enhanced drug delivery[5]. With ultrasound, sonogenic microbubbles improve transfection and intracellular delivery of macromolecules greatly[6,7]. In contrast with the existing delivery systems for gene or biotech drugs, ultrasound combined with sonogenic microbubbles can provide real-time images of soft tissue structures without ionizing radiation.

The size and concentration of microbubbles are the critical parameters that affect the scattering effect[8]. The current accepted sizes are in the range of 2 – 8 μm, which is resonant at the frequencies typically used in ultrasonic imaging (1 – 10 MHz). The concentration of 2 – 8 μm microbubbles in the microcirculation reflects the blood volume in the interesting regions, and forms the basis for the assessment of perfusion by ultrasound contrast imaging. Therefore, the concentration of 2 – 8 μm sonogenic microbubbles is considered as an index in microbubble preparation[9] and the amplitude of microbubble oscillations (and hence the scattering effect) is thus maximized[10].

Real-time ultrasound techniques combined with microbubble agent that allow for the simultaneous detection in echocardiography have been developed. Recent studies have confirmed the value of these techniques for the detection of coronary artery disease and for the definition of its severity[11,12]. Among these real-time techniques, Contrast Tuned imaging (CnTi) is a newly developed technique that can remove the clutter noise and provide real-time examinations at low mechanical index (MI). With this contrast-specific imaging mode, tissue echoes can essentially be suppressed (i. e. they appear black on the image), whereas strong echoes are generated in contrast-containing vessels.

Most commonly used phospholipids excipients are synthetic products[4], such as dipalmitoylphosphatidic acid (DPPA) and distearoylphosphatidycholine (DSPC). However, natural phospholipids are easily obtained and chemically inert compounds which have no specific interactions leading to toxic reactions or side effects. We found that preparation of sonogenic microbubbles with natural phospholipids was practicable and successful. The selected components for phospholipids-based microbubbles were hydrogenated egg phosphatidylcholine, Tween 80 and polyethylene gycol. However, we found that it was difficult to optimize them in preparation including multi-variables.

The traditional 'one-factor at a time' technique used for optimizing a multivariable system not only is time-consuming, but also may result in wrong conclusions[13]. Response surface methodology (RSM) is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors, and searching optimum conditions of factors for desirable responses. With the development of computing software, RSM has been successfully applied in many fields[14-17]. In recent years, it has been used in optimizing preparations for microparticle delivery systems[18].

This work was to use the central composite design (CCD) and RSM to evaluate the effects of three components on the concentration of 2 – 8 μm microbubbles, and to search for the optimal formulation for phospholipids-based microbubbles. To confirm the

sonogenic activity, the contrast effect *in vivo* of the optimal formulation was investigated, which would give a reliable support for the medical application of the agent.

Materials and methods

Drugs and reagents Hydrogenated egg phosphatidylcholine (EPC, >99%, Avanti Polar Lipids, USA). Tween 80 and polyethylene glycol 1500 (PEG 1500, analytical grade, Qingming Chemical Plant, China). Normal butanol (analytical grade, Beijing Chemical Plant). Perfluoropropane (electronic grade, Institute of Special Gas, Tianjin).

Instrumentation and animals New Zealand White male rabbits weighing (3 ±0.5) kg (Experiment Animal Center of 301 Hospital). An optical microscope (Olympus CX21, Japan). A coulter counter (Model BC-3000, Mindray Corporation, USA).

Sonogenic phospholipids-based microbubbles preparation The preparation of sonogenic phospholipids-based microbubbles was performed by lyophilization. The required components-EPC, Tween 80 and PEG 1500 were mixed in a test tube which was bathed in thermostatically controlled water at 65 ℃. Then normal butanol 2.5 mL was added into the tube. After all the components dissolved, the solution was transferred into a 5 mL penicillin bottle, stored in a deep freezer for 1h and lyophilized for 20 h. Approximately lyophilized sample 100 mg was saturated with perfluoropropane, sealed and injected with 0.9 mg · mL^{-1} NaCl 1 mL to form an emulsion-like solution. The concentration of 2 - 8 μm microbubbles was measured by a coulter counter.

Experimental design A series of statistically designed studies were performed previously to investigate the range in relation to different variables in phospholipids-based microbubbles preparation: EPC (5 - 10 mg), Tween 80 (15 - 25 mg) and PEG 1500 (150 - 250 mg). The components of microbubbles were arranged at different values given by CCD. Table 1 showed the design of this experiment and the corresponding results. Independent variables of the experimental design were EPC (X_1), Tween 80 (X_2) and PEG 1500 (X_3). Dependent variable was the concentration of 2 - 8 μm microbubbles (Y).

A response surface design is appropriate when the optimal region for running the process has been identified. Further optimization of phospholipids-based microbubbles preparation was carried out by using a Box-Behnken CCD with twelve star points and three replicates at center point for each of three variables.

Table 1 Results of the concentration of 2 - 8 μm sonogenic microbubbles in central composite design

Run	X_1 EPC /mg	X_2 Tween 80 /mg	X_3 PEG 1500 /mg	Y Concentration of 2 - 8 μm microbubbles/ ×10^9 · mL^{-1}
1	5	15	200	5.6
2	5	25	200	6.1
3	10	15	200	6.9
4	10	25	200	7.8
5	7.5	15	150	5.6
6	7.5	15	250	6.8
7	7.5	25	150	7.8
8	7.5	25	250	7.5
9	5	20	150	6.3
10	10	20	150	7.1
11	5	20	250	5.6
12	10	20	250	7.6
13	7.5	20	200	8.6
14	7.5	20	200	8.5
15	7.5	20	200	8.2

A second-order polynomial model obtained by a multiple regression technique for three variables using the SAS/Statistics 8.0 Package (SAS Institute Inc, USA) was adopted to describe the response surface. For three variables the equation is

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}{X_1}^2 + b_{22}{X_2}^2 + b_{33}{X_3}^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 \quad (1)$$

Where Y, predicted response, stands for the concentration of 2 - 8 μm microbubbles.

Stability test Accelerated experiment was performed to evaluate the stability of lipid-coated contrast agent. Since the phase transition temperature of lipid membrane of microbubbles was about 40 ℃, all samples in accelerated experiment were stored in well-closed 10 mL penicillin vials stored at (30 ±2)℃ with relative humidity (65 ± 5)% for six months. Morphological characteristics (observed under optical microscope), concentration and mean diameter of microbubbles (measured by coulter counter) were considered as indices. Samples of three batches were examined at 0, 1, 2, 3 and 6 months.

Contrast effect *in vivo* Contrast Tuned imaging (CnTi) technique was used to assess the sonogenic effect of the optimal fomulation selected by the response surface methodology. Five rabbits were anesthetized with intramuscular injection of pentobarbital through a 20-gauge catheter cannulated in an

ear vein. Bolus injections of the phospholipids-based microbubble were also given through the catheter (the dose of the agent of each injection was 0.1 mL·kg^{-1}). The tubing was flushed with 2 mL of saline after injection of the contrast agent. Enhancement effect in rabbit kidney parenchyma was recorded. With the same dose and process as mentioned above, SonoVue® (Bracco Inc., Italy) was used as the control.

CnTi was done using a Technos^{MPX} DU8 (Parkson, Italy). A convex probe, which has transmit/receive center frequencies of 2.63/5.26 MHz for second harmonic imaging, was used in this study. Acoustic pressure was 1 kPa. More than 15 min was allowed between injections. Ultrasound imaging was recorded on video-tapes before injection and continued until complete disappearance of the agent.

Quantitative measurements using the images recorded on videotape were made to confirm the qualitative visual findings. Changes in the video intensity of kidney parenchyma were analyzed, as time intensity curves, with a video intensity-analysis system (Tomtec P90, Tomtec Imaging Systems Inc., Bolder, CO). The image containing peak contrast enhancement was determined from the time-intensity curve, and the regions-of-interest (ROI) were set in the kidney parenchyma. The average of the pixel intensities over the ROI was analyzed by computer (Macintosh PowerBook G3, Apple Computer Inc., CA) using Adobe Photoshop® 3.0J (Adobe System Inc., CA), and the difference of maximum intensity between before and after enhancement was calculated. Duration of the contrast enhancement was also measured by time-intensity curves.

Data analysis SAS/Statistics 8.0 was used for the regression analysis of the experimental data obtained. The quality of the fit of the polynomial model equation was expressed by the coefficient of determination r^2, and its statistical significance was checked by an F-test (ANOVA). The significance of the regression coefficient was tested by a t-test.

Results and discussion

1 Optimization of the phospholipids-based microbubbles preparation

Three-dimensional response surface graphs obtained from calculated response surface are shown in Figure 1 and the two-dimensional contour plot of the model equation fitted to the data of the CCD experiment is shown in Figure 2. The response surface plot (3D) was drawn to illustrate the effects of the independent variables on the dependent ones. And these graphs were drawn imposing a constant value (i.e. the central points of the interval taken into consideration) to one of the independent variables.

The experimental results of the CCD were fitted with a second-order polynomial function. The fitted equation for estimation of the concentration of 2 – 8 μm microbubbles (Y) had the following form

$$Y = -32.263 + 2.34X_1 + 1.654X_2 + 0.13X_3 - 0.169X_1^2 - 0.031\,17X_2^2 - 0.000\,2917X_3^2 + 0.002\,4X_1X_3 - 0.001\,5X_2X_3 \quad (2)$$

From the equation above, we concluded that optimal conditions were $X_1 = 8.35$; $X_2 = 21.68$; $X_3 = 201$. The corresponding Y is 8.54×10^9 · mL^{-1}. It is shown that the signs of b_{11}, b_{22} and b_{33} are negative, so the parabola would be open downward and indicated to be of a maximum point. The model adequacy was checked by the determination coefficient r^2 and an F-test (ANOVA). $r^2 = 0.963$. Therefore, the obtained model was adequate. The degree of fit of the model was expressed by the coefficient of determination

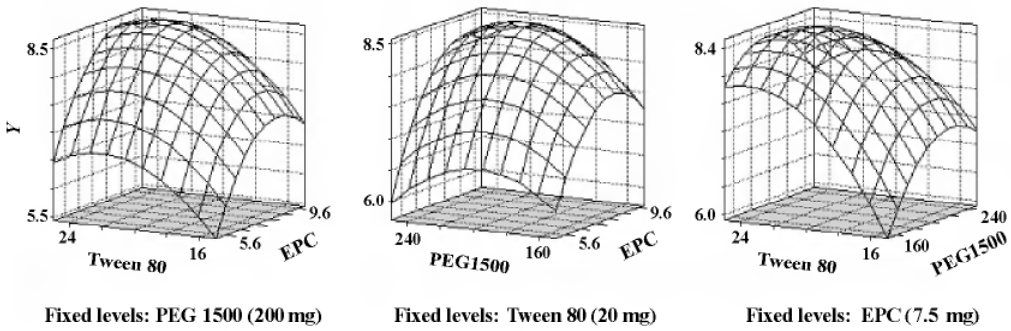

Figure 1 The response surface graph (3D) illustrating the effect of EPC, Tween 80 and PEG 1500 on the concentration of 2 – 8 μm microbubbles (Y)

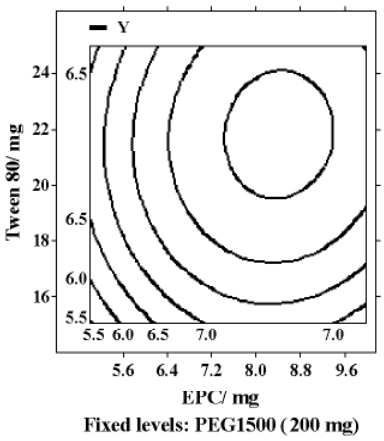

Figure 2 Two-dimensional contour plot illustrating the effect of EPC and Tween 80 on the concentration of 2 -8 μm microbubbles (Y)

r^2, which was calculated to be 0.963, indicating that 96.3% of the variability in the response could be explained by the model. The $Pr > F$ value of the polynomial model was calculated to be 0.001. The ANOVA results showed that this model is appropriate.

The results of t-test were 0.001 (constant), 0.002 (X_1), 0.001 (X_2), 0.005 (X_3), 0.001 (X_1^2), 0.003 (X_2^2), 0.004 (X_3^2), 0.098 (X_1X_3) and 0.050 (X_2X_3). The results of t-test for variance between average of observation of three-level experiment and center point showed that the difference is significant. This result indicated that optimum point was in the domain of our experiment.

This was a reconfirmation that the fitted surface had a maximum point which were EPC 8.35 mg, Tween 80 21.68 mg and PEG 1500 201 mg. The model predicted a maximum response of concentration of 2 -8 μm microbubbles $8.54 \times 10^9 \cdot mL^{-1}$ for this point. To confirm these results, experimental rechecking was performed using the optimum conditions of phospholipids-based microbubbles preparation representing this maximum point, and a mean value of the concentration of 2 -8 μm microbubbles $(8.60 \pm 0.06) \times 10^9 \cdot mL^{-1}$ ($n = 3$) was obtained. The microphotograph of the sonogenic phospholipids-based microbubbles by the optimal formulation was showed in Figure 3. The good correlation between these two results confirmed the validity of response model and the model was proven to be adequate.

2 Stability test

The results from three batches of samples ($n = 6$) had no significant difference in morphological character, microbubble concentration or mean diameter among different test conditions ($P > 0.05$). All microbubble samples in different conditions remained complete in shape and were quite stable for 6 months. The concentration of lipid-coated microbubbles reached above $2 \times 10^9 \cdot mL^{-1}$, with mean diameter about 3 μm (Figure 3). From the accelerated experiment, phospholipids-based microbubbles showed good physical stability.

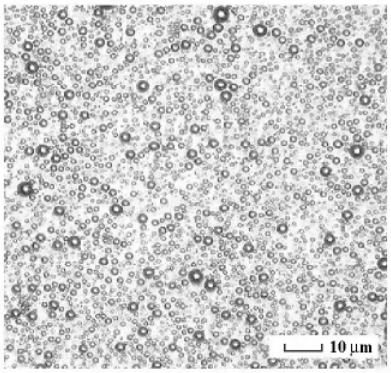

Figure 3 Microphotograph of the sonogenic phospholipids-based microbubble by the optimal formulation (×400)

3 Contrast effect *in vivo*

Contrast effect obtained from CnTi image in the kidney parenchyma was shown as Figure 4. The intensity (relative unit) and duration of the contrast effect of the optimal fomulation were 4.48 ± 0.12 and (306 ± 6) s, respectively, which showed little difference to the SonoVue® [4.28 ± 0.13, (309 ± 8) s]. From the results, it suggested that the optimal fomulation of phospholipids-based microbubbles could be developed as an effective ultrasound contrast agent for medical diagnostic use.

Conclusions

In this study, CCD combined with RSM was used for obtaining high concentration of 2 -8 μm microbubbles. From the results, all three components displayed a positive and synergistic effect on the concentration of 2 -8 μm microbubbles. The optimal condition of the phospholipids-based microbubbles preparation made the concentration of 2 -8 μm microbubbles increased to $8.60 \times 10^9 \cdot mL^{-1}$. From the

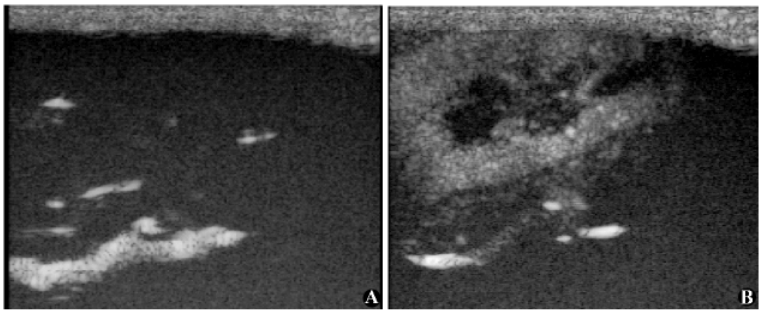

Figure 4 Contrast effect of the optimal formulation in kidney parenchyma at 1 kPa acoustic pressure. A: Before injection of phospholipids-based microbubble solution; B: After injection of phospholipids-based microbubble solution at a dose of 0.1 mL · kg^{-1}

accelerated experiment, phospholipids-based microbubbles showed good physical stability. The contrast effect *in vivo* of the optimal fomulation showed little difference to foreign market product SonoVue®, which gave a reliable confirmation for the medical application of the sonogenic phospholipids-based microbubbles.

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