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Population Bioequivalence (PBE) Statistical Method to Evaluate Particle Size Distribution of Unilamellar Liposomes Constructed by Microfluidic Chip

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Abstract

Background: Due to the high variability characteristics of liposome products and the influence of particle size on the distribution, tissue targeting behavior and clinical efficacy of liposomes, population bioequivalence (PBE) statistical method was selected to evaluate the consistency of particle size distribution of liposomes continuously prepared by microfluidic chip technology.

Method: The particle size distribution characteristics of different batches of liposome products under the same preparation process parameters in different dispersion medium (0.9% sodium chloride, 5% glucose, water) were determined and analyzed by Nanotrac Wave II, as well as the variation and stability of the particle size of liposome samples under different storage temperatures. *In vitro* PBE comparative analysis were conducted by R language programming.

Result: The particle size distribution of liposome samples prepared with pure water phase and 5% glucose under the same preparation process parameters exhibited *In vitro* population bioequivalence. The stability of liposome particle sizes prepared by 5% glucose was stable at room temperature for 10 days.

Conclusion: Through the detailed comparative study of the size distribution parameters (D10, D50, D90, PDI), PBE statistical method gives a clear evidence for the *In vitro* bioequivalence evaluation of liposome preparations, so as to better guide the *In vitro* and *In vitro* bioequivalence correlation study of liposome samples and optimization of the microfluidic process for drug-loading liposome preparation.

Keywords: Population Bioequivalence; Liposome; Particle Size Distribution; Microfluidic

Introduction

Based on the *in vivo* safety of phospholipid excipients, the development of liposome drugs using phospholipid as carrier material has been a research hotspot in the field of innovative special preparations. At present, nearly 20 liposome products have been in market for clinical use [1,2], such as Doxil, Myocet, DaunoXone, AmBisome, Abelcet, Amphotec, DepoCyte, DepoDur, EXPAREL, Visudyne, Epaxal, Marqibo, Onivyde, Vyxeos, Onpattro, Arikavce, et al. In the development of liposome products, particle size and *in vitro* dissolution are two key quality indexes that directly affect the quality stability and clinical efficacy of liposome products [3-6]. For liposome products with nanometer size (20-200nm), the common particle size detection method is based on the principle of dynamic light scattering [7-9]. D10, D50, D90, PDI and other parameters are used to describe the particle size distribution characteristics. There are many different algorithms and weighting methods for the characterization of particle size, which are expressed as Volume, Intensity, Number and Area respectively. The more consistent these values are, the better the uniformity of particle size distribution of liposome samples is.

For liposome products in clinic, FDA has successively issued official guidelines for the bioequivalence study of specific liposomes to guide the consistency evaluation of generic liposome products [2,10]. The official guideline of Doxorubicin hydrochloride

liposome [11] give the most detailed requirement of the liposome consistency *in vivo* and *in vitro*. Two important indexes for *in vitro* consistency evaluation of liposome products are particle size and dissolution. The distribution of particle size is closely related to the dissolution behavior of liposome products [3]. With respect to the evaluation of particle size consistency of liposome products, the FDA official guidance recommend the statistical method of popular bioequivalence (PBE) to evaluate the variation of particle size parameters (D10, D50, D90 and PDI) of liposome samples [12]. It is recommended to have no less than 10 sample tests in each batch and a total of no less than 30 samples particle size data for comparative analysis. Compared with the average bioequivalence (ABE) test, the PBE statistical method takes into account not only the mean value, but also the individual differences and variability within each batch. In the FDA's official guidelines, the PBE method is recommended for *in vitro* bioequivalence studies of complex preparations, such as inhaled preparations and nano preparations [13-15]. At present, there is no literature report based on PBE statistical method for the consistency evaluation of particle size distribution of liposomes prepared by microfluidic chip technique.

Microfluidic chip technology, as a new preparation process for constructing lipid nanoparticles, can significantly improve the drug loading efficiency of small-molecule drugs, protein drugs and gene drugs in lipid materials, and can effectively control the particle size range of nanoparticles [16-18]. However, the microfluidic chip has definite use times and strict cleaning process so as to ensure the quality stability and reproducibility of the liposome samples prepared between batches. Using Nanoassemblr® as an example, the manufacturer provides a disposable microfluidic chip to improve the reproducibility of lipid nanoparticles preparation, which makes the cost of liposome samples preparation higher than bulk preparation. How to effectively save the cost of the chip, achieve the continuous preparation of liposome samples, and ensure the inter-batch reproducibility of liposome samples, has become a bottleneck problem for the application of microfluidic chip technology in the liposome pilot amplification process [19-22]. In this study, liposomes with the same phospholipid formulation were prepared in different dispersion medium in a continuous manner using the microfluidic chip with classic herring bone structure. The particle size distribution variation of the liposome samples was compared using the PBE statistical method. It provides valuable data for optimizing the formulation process of liposomes and chip maintenance, guiding the *in vivo* and *in vitro* bioequivalence correlation study of liposome samples and finally controlling the development and manufacture cost.

Materials and Methods

Materials

Egg yolk lecithin (EPC) is a gift from Guangzhou Baiyunshan Hanfang Modern Pharmaceutical Co., LTD. (Lot No. A90171003). Cholesterol (CHOL) purchased from AVT (Shanghai) Pharmaceutical Technology Co., LTD. (Lot No. B80859). Anhydrous ethanol (analytical pure, AR) purchased from Chengdu Cologne Chemicals Co., LTD., Glucose (analytical pure, AR) from Sinopac Chemical Reagent Co., LTD, Sodium chloride (analytical pure, AR) from Tianjin Zhiyuan Chemical Reagent Co., LTD. Phosphotungstic acid negative staining solution (2%) purchased from Beijing Solebao Technology Co., LTD., 400 mesh copper carbon film from Suzhou Jingxi Technology Co. LTD.

Liposome Preparation

Ethanol lipid reserve solution containing 115.5mg/ mL EPC and 19.35 mg/ml CHOL (EPC:CHOL=3:1 (molar ratio)) was prepared and stored in a refrigerator at 4 °C for future use. Before the sample preparation, dilute the ethanol lipid reserve solution to 40 mg/mL total ethanol lipid, make the final liposome product with a lipid concentration of 10mg/mL, according to the flow rate ratio 3:1 of ethanol lipid phase to aqueous phase. Set the technological parameters of INano rapid nanometer preparator(INano L, Shanghai Micro&Nano Technology Inc). The total flow rate was 10ml/min, the volume ratio of aqueous phase to ethanol phase was 3:1, the total volume is 5mL. One piece of domestic microfluidic chip (GL001, Shanghai Micro&Nano Technology Inc) was used for the liposome preparation. Three different dispersive media of 0.9% NaCl (A), 5% glucose (B), and purified water (C) were selected as the aqueous phase respectively. 5 batches of blank liposome samples were prepared successively, with a total of 10 batches of samples in each dispersion medium. The liposome samples were transferred to the dialysis bag of MW3500 (MD25 dialysis bag, Shenzhen Jiemeng Cleaning Equipment Co., LTD.). and the corresponding 1000ml aqueous phase was used as the dialysate. The dialysate was stirred by magnetic stirring for 2h, and the dialysate was changed every 1 hour. The ethanol was removed, and the blank liposome samples were obtained.

MD25 dialysis bags of appropriate length were cut, fully stretched and pretreated in ultra-pure water overnight (12h). Before use, replace the ultra-pure water, put it in a drying oven (101-WB, Zhejiang Lichen Instrument Technology Co., LTD.) to be activated for 30min at 60 °C and transfer it at room temperature.

The cleaning process of microfluidic chip used the same batch of 1mL syringes before and after each sample preparation. The total flow rate was 1mL, the volume ratio of water/ethanol was 1:1, and the flow rate was 10mL/min. The same operation was repeated for three times to clean and maintain the chip.

Particle size distribution and morphology of liposomes

Liposome samples before and after dialysis were taken and not diluted. Nanotrac Wave II (Microtrac Inc., USA) was used to directly detect the sample's particle size, and SOP parameters were set as following: refractive index was 1.59, water refractive index was 1.33, detection time was 60sec/ time, and repeated for 3 times.

Take appropriate amount of the dialyzed sample, drop it on the copper network, stand it for 5-10min, and blot the sample to dry. Add an appropriate amount of 2% phosphotungstate negative dye, drain the negative dye, and wait for the sample to dry naturally. The morphology of liposomes was observed by transmission electron microscope (HT7700, HITACHI, Japan) at 100KV.

Stability Experiment

Liposome samples prepared with different dispersion medium were stored at room temperature and 4 °C, respectively, and sampled on day 0, 5, and 10. The variation of particle size distribution of liposome samples with the change of temperature and time was observed.

In vitro PBE statistical method to evaluate the particle size distribution

Blank liposome samples were prepared continuously as shown in 1.2. The measured particle size (D10, D50, D90, PDI) values of samples from different groups were examined and compared by PBE method and t-test. With reference to "FDA Guidance for Industry: Statistical Approaches for Bioequivalence (2001)", *in vitro* PBE mathematical equation were adjusted slightly. R language programming were conducted *in vitro* PBE comparative analysis.

When two batches (T: test batch; R: Reference batch) meet the following conditions, it means that the samples in two batches are bioequivalent *in vitro*, and the formula is as follows:

Reference scaling method (i.e. when $\sigma_{TR}^2 > \sigma_{TO}^2$: $\frac{(\mu_T - \mu_R)^2 + (\sigma_{TT}^2 - \sigma_{TR}^2)}{\sigma_{TR}^2} < \theta_P$;

Constant scaling method (i.e., when $\sigma_{TR}^2 < \sigma_{TO}^2$: $\frac{(\mu_T - \mu_R)^2 + (\sigma_{TT}^2 - \sigma_{TR}^2)}{\sigma_{TO}^2} < \theta_P$

Threshold value: $\theta_P = \frac{(\ln 1.25)^2 + \varepsilon_p}{\sigma_{TO}^2}$

In vitro PBE statistical method is shown in Table 1.

① Data of particle sizes from test group (T, taking the normal saline group as an example) and reference group (R, taking the water group as an example) were collected for no less than 10 samples for each group. Logarithmic transformation was performed for the response values of the sample particle size characterization parameters.

② The square of the mean difference between the two groups:

$$E0 = \hat{\Delta}^2 = (\hat{\mu}_T - \hat{\mu}_R)^2$$

95% confidence interval:

$$H0 = (\left|\hat{\Delta}\right| + t_{1-\alpha,n-s}(\frac{1}{S^2}\sum_{i=1}^{S} n_i^{-1} M_I)^{1/2})^2$$

$$M_1 = \hat{\sigma}_I^2 = \frac{1}{n_I}\sum_{i=1}^{S}\sum_{j=1}^{n_i}(I_{ij} - \bar{I}_l)^2; I_{ij} = Y_{ijT} - Y_{ijR}; n_I = (\sum_{i=1}^{S} n_i) - S$$

i is the number of batches of samples, *j* is the number of repeats of particle size test, and s is the total number of batches.

③ The data sets of the two groups are respectively represented by m and n. Only considering the numerical comparison between two groups, and the influence of sequence factors is not considered.

$$MY_T = \hat{\sigma}_T^2 = \frac{1}{n_T}\sum_{i=1}^{S}\sum_{j=1}^{n_i}(\mathrm{Y}_{Tij} - \bar{Y}_{Tl})^2; MY_R = \hat{\sigma}_R^2 = \frac{1}{n_R}\sum_{i=1}^{S}\sum_{j=1}^{n_i}(\mathrm{Y}_{Rij} - \bar{Y}_{Rl})^2;$$

$$\theta_P = \frac{(\ln 1.25)^2 + \varepsilon_p}{\sigma_{TO}^2}$$

Take $\sigma_{TO}^2 = 0.1;\ (\ln 1.25)^2 = 0.4979;\ \varepsilon_p = 0.01;\ \theta_p = 0.5079;\ \hat{\Delta} = \hat{\mu}_T - \hat{\mu}_R$

④ When the upper limit value of $\hat{\eta}$ < 0, the conclusion of population bioequivalence can be obtained. Use the following two formulas to judge.

$$\hat{\eta}_1 = \hat{\Delta}^2 + MY_T - (1+\theta_p) * MY_R,\ (\sigma_R^2 > \sigma_{TO}^2);$$

$$\hat{\eta}_2 = \hat{\Delta}^2 + MY_T - MY_R,\ \theta_p * \sigma_{TO}^2, (\sigma_R^2 > \sigma_{TO}^2);$$

Take $E0 = \hat{\Delta}^2;\ E1 = MY_T;\ E2 = -(1+\theta_p).MY_R;\ E3 = -MY_R$

$$H1 = \frac{(n-1).E1}{\chi^2_{n-1,\alpha}};\ H2 = \frac{(n-1).E2}{\chi^2_{n-1,\alpha}};\ H3 = \frac{(n-1).E3}{\chi^2_{n-1,\alpha}}$$

$U0 = (H0 - E0)^2;\ U1 = (H1 - E1)^2;\ U2 = (H2 - E2)^2;\ U3 = (H3 - E3)^2$

When $H_\eta < 0$, *in vitro* bioequivalence can be established.

Table 1: PBE statistical method to evaluate the *in vitro* bioequivalence of liposome in different dispersion medium

Result

Morphology and Particle Size of Liposome

Transmission electron microscope (TEM) was used to observe the morphology of the prepared samples. As Figure 1 shows, the blank liposome are spherical with size distribution mostly less than 50nm.

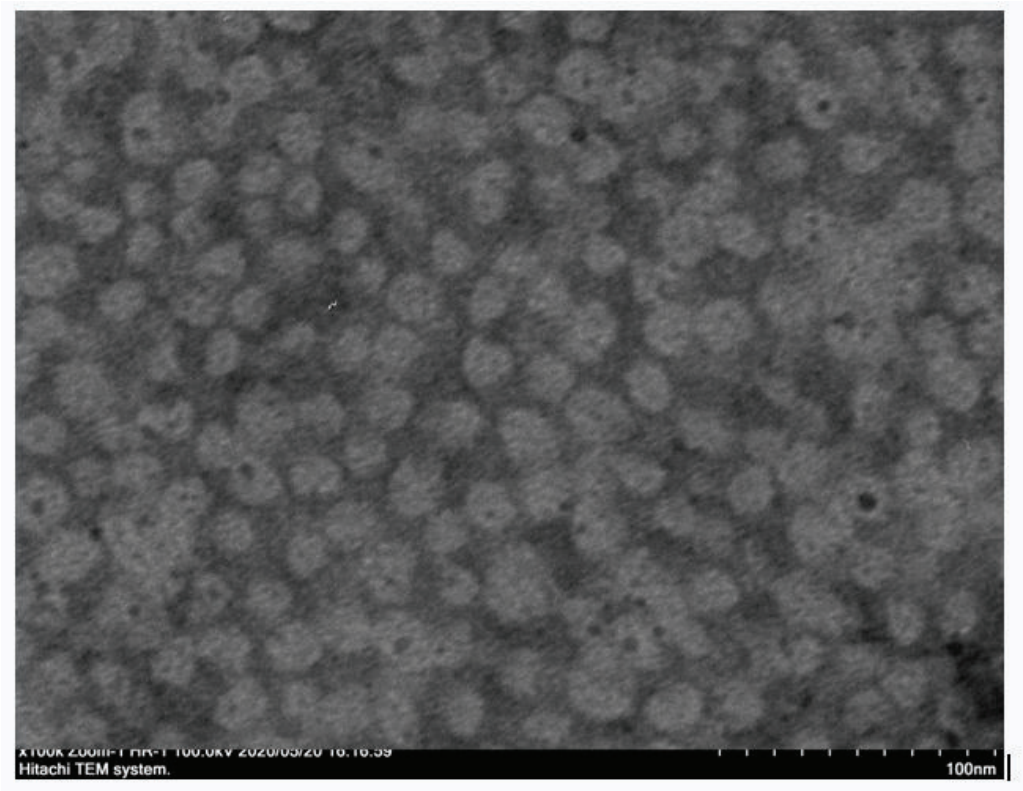

Figure 1: Morphology of blank liposomes (5% glucose) under TEM

Nanotrac WaveII nano-particle size analyzer was used to measure the particle size distribution of the prepared samples. Liposome samples were prepared continuously with normal saline (0.9% sodium chloride), 5% glucose and water dispersion medium, respectively. Table 2, Figures 2 and 3 exhibit the particle size distribution characteristics at room temperature and 4 °C. The average particle size of all the liposome samples is between 10nm and 20nm for D10, while D50 between 20nm and 30nm and D90 between 30nm and 60nm.

	A Normal Saline				B 5% Glucose				C Water			
	PDI	D10	D50	D90	PDI	D10	D50	D90	PDI	D10	D50	D90
Day 0	0.34± 0.42	15.50± 1.47	21.92± 2.29	38.40± 4.81	0.13± 0.11	17.98± 1.10	25.90± 1.70	49.45± 4.87	0.45± 0.55	17.47± 1.35	25.28± 2.11	48.35± 5.99
Day 5, 4 °C	0.44± 0.16	15.38± 1.15	22.22± 1.96	38.04± 4.82	0.58± 0.94	17.33± 2.24	27.41± 6.24	55.10± 11.71	0.43± 0.31	16.70± 1.19	24.30± 1.61	45.53± 3.69
Day 10, 4 °C	0.55± 0.5	15.90± 1.08	22.67± 1.79	41.83± 4.94	0.74± 0.68	18.30± 0.98	26.19± 1.47	54.91± 11.19	1.08± 1.11	17.21± 1.53	25.16± 2.33	54.8± 10.68
Day 5, 37 °C	0.44± 0.17	15.50± 1.00	22.19± 1.39	40.10± 3.61	0.32± 0.18	18.89± 1.13	26.89± 2.23	50.937± 6.56	0.41± 0.16	17.34± 1.42	25.16± 1.82	46.29± 2.50
Day 10, 37 °C	0.95± 0.98	16.50± 1.21	23.79± 2.32	46.57± 8.86	0.50± 0.18	18.58± 1.03	26.39± 1.76	52.67± 4.92	0.26± 0.25	18.08± 1.38	25.89± 2.03	46.10± 4.80

Table 2: Particle size distribution characteristic of liposome prepared under different dispersion medium (Mean±SD)

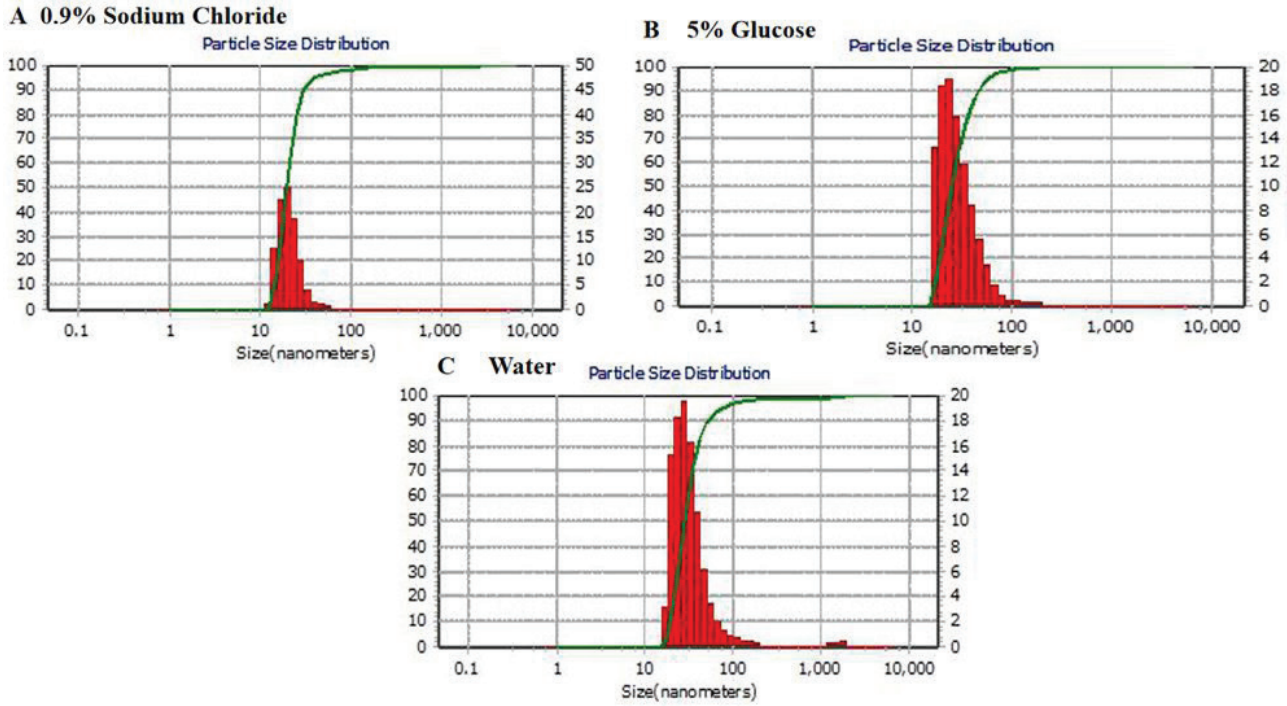

Figure 2: Particle size distribution of liposome samples prepared by different aqueous phases **(A)** Normal saline; **(B)** 5% glucose; **(C)** Water

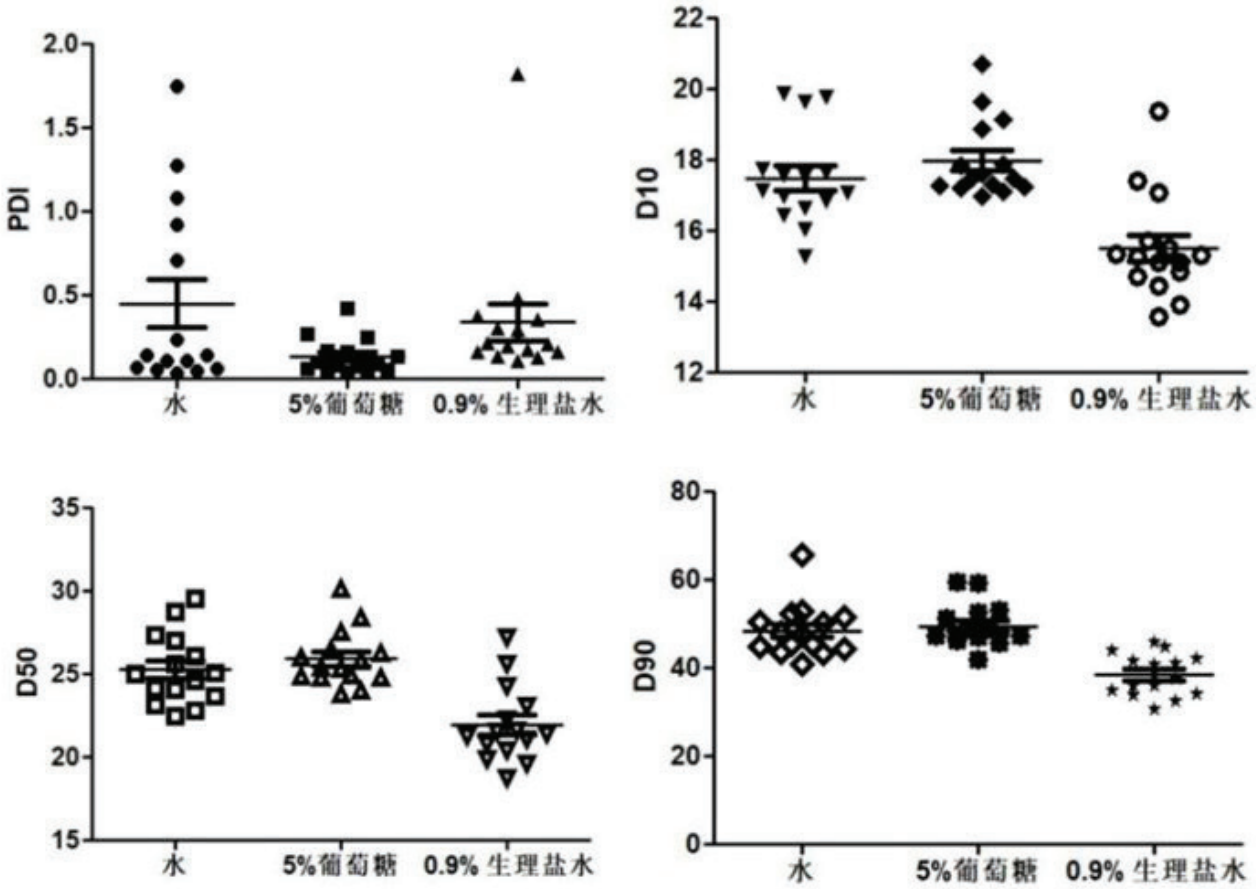

Figure 3: Particle size distribution of liposome samples in different aqueous phases (Day 0) **(A)** Normal saline; **(B)** 5% glucose; **(C)** Water

The particle size stability of liposome samples

Liposome samples prepared from 5% glucose were placed at room temperature and 4 °C for 0, 5 and 10 days, and the change of particle size distribution of D10, D50, D90 and PDI with time was shown in Figure 4.

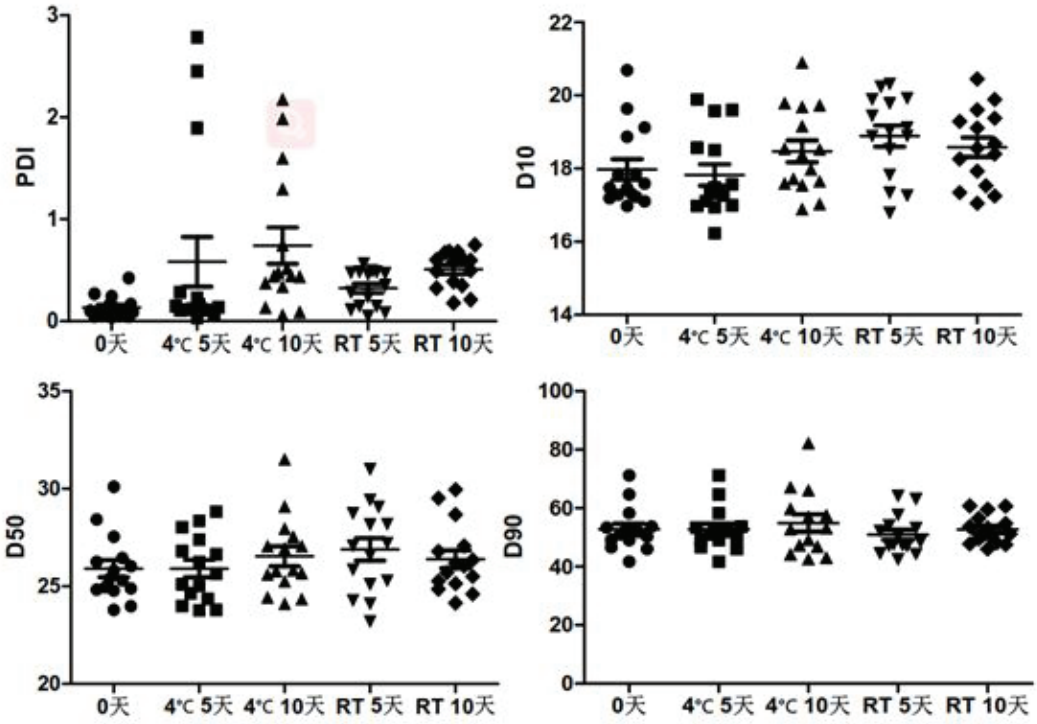

Figure 4: Particle size changes of liposome samples containing 5% glucose at 4 °C and room

Comparative study on consistency of particle size distribution of liposome samples`

t-test: The significant difference of particle size distribution of liposome samples prepared by different dispersion phases (0 days) was analyzed by unpaired t-test, and the results were shown in Table 3. Only the PDI values of group A (normal saline) to Group B (5% glucose) and Group B to Group C (water) showed significant differences (P<0.05), while the particle size distribution of liposome samples between group A and Group C showed no significant differences.

	A VS B	A VS C	B VS C
PDI	P<0.05	NS	P<0.05
D10	NS	NS	NS
D50	NS	NS	NS
D90	NS	NS	NS

Table 3: Statistic analysis of particle size distribution of liposome samples (Day 0) in different dispersion medium **(A)** Normal saline; **(B)** 5% glucose; **(C)** Water by t-test

PBE Statistical Analysis: PBE statistical method was used to analyze the particle size distribution of liposomes prepared with normal saline (group A), 5% glucose (group B), and water (group C) .When H_{η} is less than 0, it means that different aqueous medium have no influence on the size distribution of liposomes under the same preparation conditions. Different aqueous medium can be replaced with each other. PBE statistical results are presented in Table 4. The particle size distribution of liposome samples prepared with pure water phase and 5% glucose under the same preparation process parameters has *in vitro* population bioequivalence.

		B VS C	A VS C	A VS B
PDI	$H_{\eta 1}$	-2.712041	-2.540546	1.803148
	$H_{\eta 2}$	/	/	/
D10	$H_{\eta 1}$	/	/	0.035389
	$H_{\eta 2}$	-0.046004	-0.028715	/
D50	$H_{\eta 1}$	0.007334	0.035918	0.039295
	$H_{\eta 2}$	/	/	/
D90	$H_{\eta 1}$	-0.01063	0.042281	0.069614
	$H_{\eta 2}$	/	/	/

Table 4: Consistency comparison of particle size distribution of liposomes prepared from different aqueous phases by PBE statistical analysis method

Discussion

At present, Nanoassemblr® microfluidic apparatus are equipped with clear requirements for the matching of the original microfluidic chip. To ensure the uniformity and reproducibility of the particle size distribution of the nanoparticles prepared by microfluidic chip, the use times and the chip cleaning process are explicitly limited. In this study, EPC/CHOL liposome samples were prepared using a domestic microfluidic chip with a classical herring bone structure, and the chips were cleaned according to the cleaning process before sample preparation. More than five batches of liposomes were prepared, and the particle size distributions of liposomes in different aqueous phase were compared. The durability of the microfluidic chip was investigated under the conditions of 10mg/ml lipid concentration and 10ml/min flow rate. After more than 5 times of continuous liposome sample preparation, the internal structure of the microfluidic chip was observed under a microscope, as shown in Figure 5. The herringbone structure of the chip before use was clear and tractable, and there was no externally introduced residue or crack in the groove and pipe. After dozens of times using, the chip herringbone groove appears liquid residue, pipe edge cracks. This indicates that the microenvironment inside the chip will be worn after repeated flushing by the fluid, but the particle size of the liposome samples prepared in the 6th continuous process is also less than 100nm. The chip's eddy capability will not be affected.

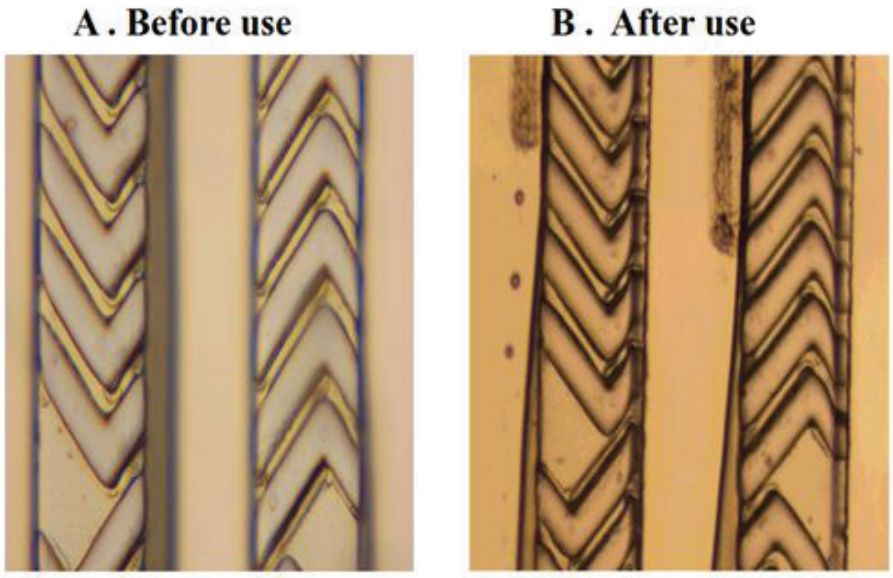

Figure 5: The internal structure of microfluidic chip

The existing microfluidic chips emphasized the sample cleaning process before and after each sample preparation. In addition, the using times of chip should be no more than 20 times so as to ensure the uniformity and stability of particle size distribution of the liposome samples following the instruction. In this study, the whole experiment was done on the same chip. Lipid formulations were optimized, and liposome samples of different aqueous phases were prepared continuously, and the differences in particle size distribution of in-batch and inter-batch samples were compared. The results showed that the stability of liposome samples was investigated at room temperature and 4 °C for 10 days, and the stability of liposome particle sizes prepared by different aqueous phases was significantly different.

For the liposome samples in this study, the particle size uniformity, reproducibility and stability were the best when the lipid concentration of ethanol was 40mg/ mL, the volume ratio of aqueous phase/ethanol phase was 3:1, and the flow rate was 10ml/min. It also shows that the selection of different aqueous phase (formulation factor) can affect the frequency of chip use and durability.

As a innovative complicate preparation, the PK/PD behavior of liposome drugs *in vivo* is closely related to the physiochemical characteristics. For the quality control, particle size and dissolution are two key characterization factors. The release of liposome-encapsulated drugs under different conditions was closely related to the size distribution of liposome. The distribution of particle size directly affects the pharmacokinetics, biological distribution and stability of liposome products, which then influence the clinical therapeutic effect of drugs on patients and lead to the serious adverse reactions. Through PBE statistical method to analyze the particle size distribution characteristics of liposome samples with different prescriptions, the process optimization of liposome preparation can be conducted by an effective application of QbD concept. PBE was selected to make a comparative analysis of the consistency of particle size distribution of liposome samples, that taking into full consideration on the possible inter-sample variability caused by particle residue in microfluidic chip after the continuous sample preparation process. It is also used to further verify the durability of the chip under the lipid formulation process conditions. Different lipid formulations directly affect the quality uniformity of lipid samples, as well as the frequency of use and durability of microfluidic chips.

Conclusion

Through the detailed comparative study of the size distribution parameters (D10, D50, D90, PDI), PBE statistical method gives a clear evidence for the *in vitro* bioequivalence evaluation of liposome preparations, so as to better guide the *in vivo* and *in vitro* bioequivalence correlation study of liposome samples and optimization of the microfluidic process for drug-loading liposome preparation.

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