Annexure A

Annexure contains the abstracts and posters as presented at the 7th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology from 8 – 11 March 2010, Valletta, Malta. I was author of both abstracts and poster and only presenter of one poster: The size determination of Pheroid™ formulations and liposomes.

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SIZE CHARACTERIZATION OF PHEROID™ FORMULATIONS AND LIPOSOMES

C. Slabbert, L. Nieuwoudt, L.H. du Plessis and A.F. Kotzé

Unit of Drug Research and Development, Faculty of Health Science, North-West Unversity, Potchefstroom, 2531, South Africa

INTRODUCTION

Pheroid™ formulations consist of essential fatty acid dispersed in a liquid and nitrous oxide gas phase. Fatty acids are natural ingredient of the body compared to artificial polymer of other lipid based delivery systems. Pheroid™ formulations can entrap both water and lipid soluble compounds with high efficacy. Low toxicity to the ingredients, drug protection, higher volume of distribution and increse efficacy is some of the advantages of Pheroid™ technology. Size is dependent on the formulation ranging from 200 nm to 50 µm in diameter (Grobler et al. 2007).

Liposomes are reputable as a membrane system valuable for the delivering of a vast amount of materials. Liposome formulations has the advantage of larger dose loads, slower release, reduced toxicity, target specific delivery with increase efficacy. This colloidal drug delivery system, dependent on the manufacturing procedure, vary in diameter size from 80 nm to 100 µm (New 1990). Disadvantage include the toxicity of some formulations, low entrapment ability and high cost (Mozafari 2005).

Particle size, internal aqueous volume and chemical composition are important parameters affecting the biochemical properties of drug delivery systems (Sato et al. 2006). The size of the different Pheroid™ and liposome formulations were determined by flow cytometry, confocal laser scanning microscopy and with a Malvern Mastersizer.

EXPERIMENTAL METHOD

Pheroid™ preparation

Pheroid™ vesicles and Pheroid™ microsponges were prepared according to patented formulations. Pheroid™ vesicles and Pheroid™ microsponges consist of the same ingredients, except for the addition of fatty acids with longer aliphatic tails in the case of Pheroid™ microsponges.

Liposome preparation

Six different liposome formulations (L01 to L06) were manufactured using different methods or ingredients (See Table 1) . Liposome formulations (L01 to L05) were prepared by the film hydration method. The combination of cholesterol (CH), phospatidylcholine (PC) and/or phospatidyl glycerol (PG) (Sigma Aldrich®) were dissolved in a chloroform:methanol (2:1 v/v) mixture. The organic phase were removed under reduced pressure to form thin film on the flask. The film was dispersed in phosphate buffer solution. Liposome formation was attained by rotation with glass beads, sonication or a combination of both. Reverse-phase evaporation vesicles (L06) was manufactured by dissolving the lipids in a mixture of organic and aqueous phase. The organic phase was slowly remove by evaporation to form a gel.

Table 1 Different liposome formulations

Nr.	Method	Ingredients		
		CH	PC	PG
L01	Film hydration, no sonication	×	×	
L02	Film hydration, sonication (bath)	×	×	
L03	Film hydration, sonication (rod)	×	×	
L04	Film hydration, sonication (rod)	×	×	×
L05	Film hydration, no sonication	×	×	×
L06	Reverse phase liposomes	×	×	

Size determination

Flow cytometry analysis

Forward laser light scatter (FSC) analysis of the formulations was performed using a FACSCalibur™ equipped with a 488 nm argon ion laser linked to CellQuest Pro. Data were collected by acquiring 100 000 events and analysed with FCS Express V3 software. Size calibration beads were analysed as standard yielding a histogram depicting relative particle size ranges. Samples were analysed according to these standard.

Malvern Mastersizer

The mean particle size, distribution and uniformity of the formulations were measured by dynamic light

scattering analysis using a Malvern Mastersizer 2000 equipped with a blue solid light laser.

Confocal laser scanning microscopy (CLSM)

Images were captured on a CLSM equipped wit a He/Ne red laser and a argon green laser. EZ-C1 software was used for the analysis of samples. Samples were stained with a 1 mg/ml Nile Red in DMSO solution for 15 minutes prior to analysis.

RESULTS AND DISCUSSION

Size distribution of the formulations were quantitatively analysed with flow cytometry and Malvern Mastersizer (See Table 2). Superior uniformity was found with formulation L01 and L04. Smaller size diameter indexes were seen with Pheroid™ vesicles, Pheroid™ microsponges and liposome formulation L02.

Table 2 Result from size analysis

Formulation	Mean diameter (µm)	Size diameter (µm)	Uniformity
L01	9.820	0.4-141	0.99
L02	11.522	0.35-60	0.686
L03	2.094	0.28-110	1.26
L04	14.398	0.35-200	0.958
L06	45.159	0.4-225	0.736
Vesicles	1.939	0.35-56	1.3
Microsponges	1.558	0.25-56	1.32

Confocal images showed higher uniformity with the sonicated liposomes. Pheroid™ vesicles and Pheroid™ microsponges showed superior structural uniformity.

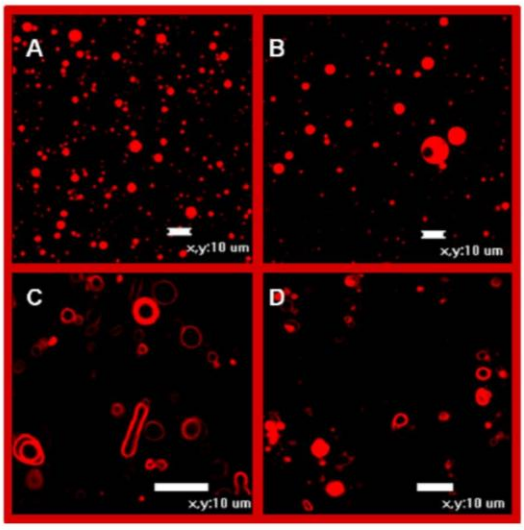

Figure 1 Micrographs of Pheroid™ vesicles (A), Pheroid™ microsponges (B), L04 (C) and L06 (D).

CONCLUSION

Formulations showed size distribution of between 0.25 µm and 225µm depending on the manufacturing procedure and ingredients. Liposomes can be manipulated to give a required size. Pheroid™ vesicles and Pheroid™ microsponges have the same size distribution as certain liposomes. The natural ingredients of Pheroid™ formulations makes this an attractive alternative drug delivery system.

REFERENCES

[1] Grobler, A.F., Kotze, A., and Du Plessis, J., 2007. The design of a skin-friendly carrier for cosmetic compounds using Pheroid™ technology. In: Wichers, J.W. (Ed.), Delivery system technologies. Allured Publishing Corporation, Wheaton, IL, pp. 283-311.

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Size characterization of Pheroid™ formulations and liposomes

C. SLABBERT, L. NIEUWOUDT, L.H. DU PLESSIS AND A.F. KOTZÉ

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Liposomes are reputable as a membrane system valuable for the delivering of a vast amount of materials. Liposome formulations has the advantage of larger dose loads, slower release, reduced toxicity, target specific delivery with increase efficacy. This colloidal drug delivery system, dependent on the manufacturing procedure, vary in diameter size from 80 nm to 100 µm (New 1990). Disadvantage include the toxicity of some formulations, low entrapment ability and high cost (Mozafari 2005).

Particle size, internal aqueous volume and chemical composition are important parameters affecting the biochemical properties of drug delivery systems (Sato et al. 2006). The size of the different Pheroid™ and liposome formulations were determined by flow cytometry, confocal laser scanning microscopy and with a Malvern Mastersizer.

Methods

Pheroid™ preparation

Pheroid™ vesicles and Pheroid™ microsponges were prepared as reported in Du Plessis *et al., 2009*. Pheroid™ vesicles and Pheroid™ microsponges consist of the same ingredients, except for the addition of fatty acids with longer aliphatic tails in the case of Pheroid™ microsponges.

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Six different liposome formulations (L01 to L06) were manufactured using different methods or ingredients (See Table 1) . Liposome formulations (L01 to L05) were prepared by the film hydration method. The combination of cholesterol (CH), phospatidylcholine (PC) and/or phospatidyl glycerol (PG) (Sigma Aldrich®) were dissolved in a chloroform:methanol (2:1 v/v) mixture. The organic phase were removed under reduced pressure to form thin film on the flask. The film was dispersed in phosphate buffer solution. Liposome formation was attained by rotation with glass beads, sonication or a combination of both. Reverse-phase evaporation vesicles (L06) was manufactured by dissolving the lipids in a mixture of organic and aqueous phase. The organic phase was slowly remove by evaporation to form a gel.

Table 1 ~ Different liposome formulations

Nr.	Method	Ingredient CH	PC	PG
L01	Film hydration, no sonication	X	X	
L02	Film hydration, sonication (bath)	X	X	
L03	Film hydration, sonication (rod)	X	X	
L04	Film hydration, sonication (rod)	X	X	X
L05	Film hydration, no sonication	X	X	X
L06	Reverse phase liposomes	X	X	

Size determination ~ Flow cytometry analysis

Forward laser light scatter (FSC) analysis of the formulations was performed using a FACSCalibur™ equipped with a 488 nm argon ion laser linked to CellQuest Pro. Data were collected by acquiring 100 000 events and analysed with FCS Express V3 software. Size calibration beads were analysed as standard yielding a histogram. Relative size ranges were determined by calculating the linear regression yielding a equation y=mx+b. Samples were analysed according to this standard.

Size determination ~ Malvern Mastersizer

The mean particle size, distribution and uniformity of the formulations were measured by dynamic light scattering analysis using a Malvern Mastersizer 2000 equipped with a blue solid light laser.

Confocal laser scanning microscopy (CLSM)

Images were captured on a CLSM equipped wit a He/Ne red laser and a argon green laser. EZ-C1 software was used for the analysis of samples. Samples were stained with a 1 mg/ml Nile Red in DMSO solution for 15 minutes prior to analysis.

Results and Discussion

Forward scatter (FSC) analysis of the size calibration beads gave distinct peaks as seen in Figure 1. Correlation between the geometric mean values and size were analysed to give a correlation graph (Figure 2) with an equation of y=1.607x+0.4496 and a correlation value of $r^2 = 0.9781$. This mathematical equation were use to divide the forward scatter values into ranges or gates representing different size ranges. Size distribution were determined quantitatively and is displayed in Table 2. Evaluation of dot plots showed a distinct difference in the population of the Pheroid™ vesicles and liposome formulation (Figure 3). Dynamic laser light scattering was use to evaluate the size range of the formulations and is shown in Table 2. Figure 4 shows the difference in size evaluation between the Pheroid™ vesicles and L04 liposome formulation. Correlation between the flow cytometric evaluation and Malvern analysis was low. This can be because of the irregular shape and structure of the liposomes (Data not shown). Pheroid™ vesicles showed a correlation of $r^2 = 0.851$ between the different analysis methods (Figure 5). Confocal images showed higher uniformity with the sonicated liposomes. Pheroid™ vesicles and Pheroid™ microsponges showed superior structural uniformity.

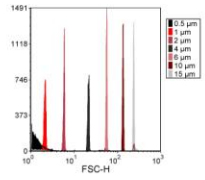

Figure 1 ~ Histogram of the FSC-values of size calibration beads

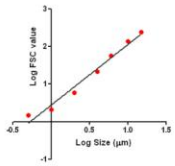

Figure 2 ~ Size calibration curve

Table 2 ~ Size distribution of the different formulations

Formulation	Size distribution FACS	Size distribution Malvern
L01	0.1 - 38.39µm	0.399 - 83.11 µm
L02	0.1 – 40.17 µm	0.399 – 82.365 µm
L03	0.1 – 34.19 µm	0.399 - 40.414 µm
L04	0.1 – 25.05 µm	0.399 – 57.347 µm
L05	0.1 – 29.34 µm	0.399 – 44.091 µm
L06	0.1 – 31.40 µm	0.356 – 63.669 µm
Pheroid™ Vesicles	0.1 -12.34 µm	0.356 – 12.619 µm
Pheroid™ Microsponges	0.1 – 10.61 µm	0.159 – 12.619 µm

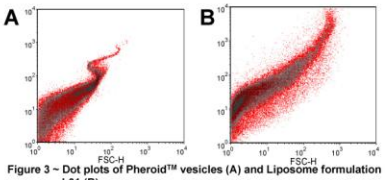

Figure 3 ~ Dot plots of Pheroid™ vesicles (A) and Liposome formulation L01 (B)

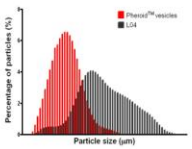

Figure 4 ~ Malvern size evaluation of Pheroid™ vesicles and L04

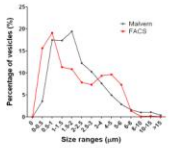

Figure 5 ~ Correlation between Malvern and FACS analysis of Pheroid™ vesicle

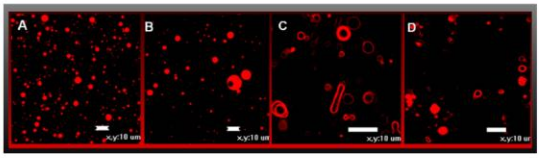

Figure 6 ~ Micrographs of Pheroid™ vesicles (A), Pheroid™ microsponges (B), L04 (C) and L06 (D)

Conclusion

Formulations showed size distribution of between 0.16 and 83 µm for Malvern and 0.1 and 41 µm for FACS analysis. Size distribution is dependent on the manufacturing procedure and ingredients. Liposomes can be manipulated to give a required size. Pheroid™ vesicles and Pheroid™ microsponges have the same size distribution as certain liposomes. The natural ingredients of Pheroid™ formulations makes this an attractive alternative drug delivery system.

Reference

[1] Grobler, A.F., Kotze, A., and Du Plessis, J., 2007. The design of a skin-friendly carrier for cosmetic compounds using PheroidTM technology. In: Wichers, J.W. (Ed.), Delivery system technologies. Allured Publishing Corporation, Wheaton, IL, pp. 283-311.

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ENTRAPMENT ABILITY OF PHEROID™ FORMULATIONS AND LIPOSOMES

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Unit of Drug Research and Development, Faculty of Health Science, North-West Unversity, Potchefstroom, 2531, South Africa,

INTRODUCTION

Drug delivery systems can be used to improve the efficacy, specificity and tolerability of compounds Liposomes, a colloidal drug carrier has a diameter ranging from 80 nm to 100 µm (Date et al. 2007). These vesicles have an aqueous volume that is enclosed by lipid membrane consisting of a combination of cholesterol and phospholipids. Liposomes can vary in size according to the method used during manufacturing. Liposomes have the ability to entrap both lipid and water soluble compounds (New 1990).

Pheroid™ formulations are patented colloidal delivery systems consisting of essential fatty acids that are natural ingredient of the body. Lipids are dispersed in a liquid and nitrous oxide gas phase (Grobler et al. 2007). Pheroid™ technology consist of different formulations depending on the composition and method of manufacturing (Uys 2006). The unique composition of Pheroid™ gives elasticity and fluidity to the vesicles and microsponges formulations. The Pheroid™ formulation differs in size ranging from a diameter of 200 nm to 50 µm. This drug carriers can entrap hydrophilic and lipophilic compounds (Grobler et al. 2007).

To determine the entrapment ability of Pheroid™ and liposomes, fluorescent labelled proteins were entrapped and analysed with flow cytometry and confocal laser scanning microscopy.

EXPERIMENTAL METHOD

Liposome preparation

Egg-phosphotadylcholine cholesterol liposomes were prepared by the film hydration method. Phosphatidyl glycerol, phosphatidylcholine and cholesterol (Sigma Aldrich®) were dissolved in the organic phase consisting of chloroform and methanol. The organic phase was evaporated under reduced pressure to form a thin film on the flask. The film was hydrated with a phosphate buffer (pH 7.4), containing labelled proteins. Liposomes formation was attained by rotation with glass beads (2mm). Unilaminer vesicles were obtained by sonication using an ultrasonic rod (New 1990). Samples were taken before and after sonication..

Pheroid preparation

Pheroid™ vesicles and microsponges were prepared according to the patented formulations. Proteins were added at time zero. Samples were taken and measured after a 15 minutes incubation period with Alexa Fluor 430 and measured at time intervals of 1, 3, 6, 9, 12 and 24 hours.

Fluorescence Labelling

Proteins were labelled with Alexa Fluor 430 (Molecular probes) prior to analysis. Liposomes and Pheroid™ formulations were stained with a 1 mg/ml Nile Red in DMSO solution (Sigma Aldrich®) with an incubation period of 15 minutes.

Flow cytometry analysis

Analysis was preformed using a FACSCalibur™ equipped with a 488 nm argon ion laser linked to CellQuest Pro software for analysis. Forward scatter, side scatter, FL1 and FL3 were set to logarithmic amplification. Data were analysed using FCS Express V3 software. Samples were diluted with PBS (pH 7.4) and a total of 100 000 events were counted for each sample. Samples were run in duplicate.

Confocal laser scanning microscopy (CLSM)

Images were captured on a CLSM equipped with a He/Ne red laser and a argon green laser. EZ-C1 software was used for the qualitatively analysis of samples in duplicate.

RESULTS AND DISCUSSION

The entrapment ability of liposomes and Pheroid™ formulations were determined qualitatively and quantitatively. CLSM results showed the presence of labelled proteins in the Pheroid™ formulations with an increases over time. Figure 1 illustrates the protein entrapped Pheroid™ vesicles at three time

intervals. Liposomes also showed entrapment of Proteins to a lesser extend (Figure 1).

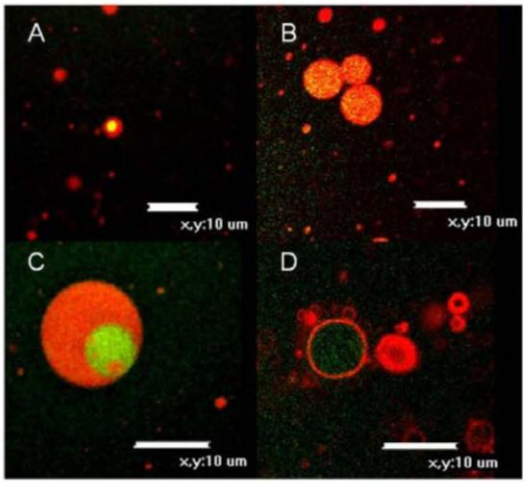

Figure 1 Micrographs of Pheroid™ vesicles at time intervals of 1 hour (A), 6 hours (B) and 12 hours (C) and liposomes (D) with entrapped labelled protein. The green represents the labelled proteins and the Pheroid™ vesicles and liposomes are red in colour.

The entrapment capacity of liposomes and Pheroid™ were analysed qualitatively by flow cytometry using a FSC/FL1 plot. All samples were compared to the control of unstained liposomes and Pheroid™ respectively. Results showed a higher entrapment ability of Pheroid™ over time with a 59% trapping efficacy of Pheroid™ vesicles after 24 hours (Figure 2). Pheroid™ microsponges also showed higher entrapment ability over time. Unilaminer liposomes gave an entrapment efficacy of 33%. The liposomes measured before sonication showed higher entrapment ability of 59% (Figure 3). This can be due to the size difference between the two liposome samples.

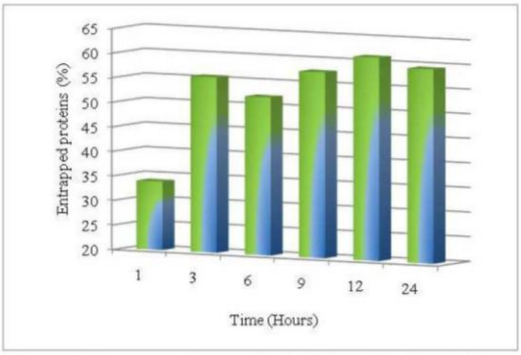

Figure 2 Entrapped proteins in Pheroid™ vesicles over time.

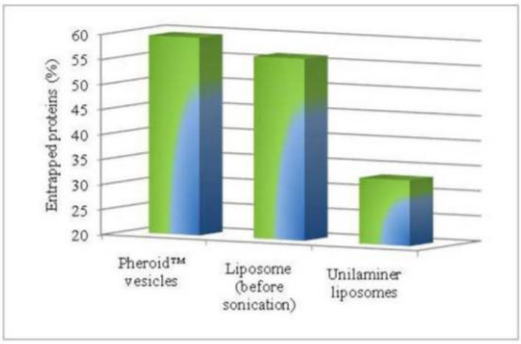

Figure 3 Entrapped proteins in Pheroid™ vesicles (24 hours) and liposomes before and after sonication.

CONCLUSION

Pheroid™ vesicles and microsponges' entrapment ability increased over time. Pheroid™ vesicles at 24 hours showed a 26% higher entrapment efficacy compared to liposomes. Pheroid™ formulations have the advantage to entrap proteins after manufacturing. This can be an advantage especially to other compounds with low stability in solution.

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[1] Date, A.A., Joshi, M.D., and Patravale, V.B., 2007. Parasitic diseases: Liposomes and polymeric nanoparticles versus lipid nanoparticles. Adv. Drug Deliv. Rev. 59, 505-521.

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RESULTS AND DISCUSSION

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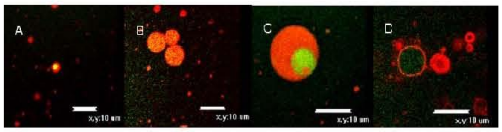

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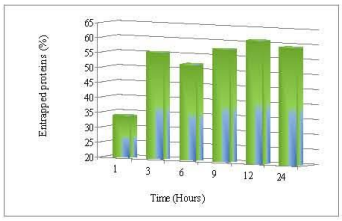

Figure 2: Entrapped proteins in Pheroid™ vesicles over time.

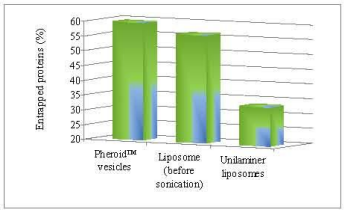

Figure 3: Entrapped proteins in Pheroid™ vesicles (24 hours) and liposomes before and after sonication.

CONCLUSION

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Annexure B

Results obtained from the physical stability, size, pH and entrapment efficacy during the experimental period of three months under all employed conditions and 14 day entrapment efficacy of different Pheroid™ vesicle formulations

List of tables

Table 1 – Three month stability testing of span and pH of Pheroid™ vesicles at 5°C.

Week	Span					pH				
	1	2	Ave	SEM	p-value	1	2	Ave	SEM	p-value
0	3.06	3.08	3.07	0.01	-	6.55	6.53	6.54	0.01	-
1	2.72	2.73	2.73	0.01	0.732	6.09	6.02	6.06	0.04	2.896
2	3.56	2.73	3.15	0.41	1.000	6.06	6.26	6.16	0.10	0.960
3	2.87	2.79	2.83	0.04	0.924	5.91	6.25	6.08	0.17	0.915
4	2.71	2.65	2.68	0.03	0.630	5.74	6.68	6.21	0.47	0.980
8	3.10	2.97	3.04	0.06	1.000	5.89	6.79	6.34	0.45	0.999
12	3.16	3.06	3.11	0.05	1.000	5.77	6.59	6.18	0.41	0.970

Table 2 – Three month stability testing of span and pH of Pheroid™ vesicles at 25°C.

Week	Span					pH				
	1	2	Ave	SEM	p-value	1	2	Ave	SEM	p-value
0	3.06	3.08	3.07	0.01	-	7.92	7.94	7.93	0.01	-
1	4.27	2.75	3.51	0.76	0.966	7.24	7.00	7.12	0.12	*0.019*
2	2.72	2.75	2.73	0.02	0.991	7.41	7.03	7.22	0.19	*0.037*
3	2.70	2.66	2.68	0.02	0.981	7.26	7.02	7.14	0.12	*0.022*
4	2.72	2.68	2.70	0.02	0.985	7.17	7.01	7.09	0.08	*0.016*
8	4.10	2.99	3.54	0.55	0.952	5.81	5.58	5.70	0.12	*0.001*
12	3.03	2.96	3.00	0.03	1.000	5.10	4.86	4.98	0.12	*0.001*

Table 3 – Three month stability testing of span and pH of Pheroid™ vesicles at 30°C.

Week	Span					pH				
	1	2	Ave	SEM	p-value	1	2	Ave	SEM	p-value
0	3.06	3.08	3.07	0.01	-	6.67	6.63	6.65	0.02	-
1	4.30	4.35	4.33	0.02	*0.001*	6.99	7.00	7.00	0.01	*0.005*
2	2.79	2.76	2.78	0.02	*0.004*	6.83	6.70	6.77	0.07	0.454
3	2.67	2.75	2.71	0.04	*0.001*	6.47	6.33	6.40	0.07	*0.027*
4	2.73	2.75	2.74	0.01	*0.002*	6.13	6.14	6.14	0.01	*0.001*
8	2.97	2.92	2.94	0.02	0.225	4.91	4.86	4.89	0.03	*0.001*
12	3.09	2.96	3.02	0.07	0.946	3.44	3.49	3.47	0.03	*0.001*

Table 4 – Three month stability testing of span and pH of Pheroid™ vesicles at 37°C.

Week	Span					pH				
	1	2	Ave	SEM	p-value	1	2	Ave	SEM	p-value
0	3.06	3.08	3.07	0.01	-	6.65	6.63	6.64	0.01	-
1	4.3	4.11	4.21	0.10	*0.002*	6.99	6.91	6.95	0.04	0.998
2	2.86	2.85	2.85	0.01	0.805	6.83	6.13	6.48	0.35	1.000
3	2.84	2.80	2.82	0.02	0.696	6.47	5.56	6.02	0.46	0.933
4	2.47	3.01	2.74	0.27	0.442	6.13	5.08	5.61	0.53	0.641
8	3.09	3.20	3.15	0.05	0.998	4.91	3.30	4.11	0.81	*0.041*
12	3.37	3.45	3.41	0.04	0.414	3.44	2.96	3.20	0.24	*0.008*

Table 5 – Three month stability testing of span and pH of liposomes at 5°C.

Week	Span					pH				
	1	2	Ave	SEM	p-value	1	2	Ave	SEM	p-value
0	6.47	6.45	6.46	0.01	-	7.39	7.40	7.40	0.01	-
1	9.02	8.25	8.64	0.38	0.991	7.08	7.19	7.14	0.06	0.389
2	8.71	13.43	11.07	2.36	0.792	7.20	7.38	7.29	0.09	0.962
3	10.57	12.87	11.72	1.15	0.694	7.19	7.35	7.27	0.05	0.920
4	11.43	10.50	10.096	0.46	0.807	7.14	7.32	7.23	0.09	0.785
8	8.41	16.27	12.34	3.93	0.597	7.11	7.29	7.20	0.09	0.656
12	9.50	17.42	13.46	3.96	0.433	7.04	7.28	7.16	0.12	0.483

Table 6 – Three month stability testing of span and pH of liposomes at 25°C.

Week	Span					pH				
	1	2	Ave	SEM	p-value	1	2	Ave	SEM	p-value
0	6.47	6.45	6.46	0.01	-	7.33	7.38	7.36	0.03	-
1	9.27	9.01	9.14	0.13	0.603	6.87	6.88	6.88	0.01	0.385
2	10.07	6.58	8.33	1.74	0.864	6.95	6.87	6.91	0.04	0.456
3	9.63	8.58	9.11	0.53	0.615	6.47	6.53	6.5	0.03	*0.050*
4	10.25	11.27	10.76	0.51	0.194	6.32	6.42	6.37	0.05	*0.025*
8	7.41	8.31	7.86	0.45	0.957	5.69	5.50	5.60	0.1	*0.001*
12	8.20	12.34	10.27	2.07	0.282	5.42	4.65	5.04	0.39	*0.001*

Table 7 – Three month stability testing of span and pH of liposomes at 30°C.

Week	Span					pH				
	1	2	Ave	SEM	p-value	1	2	Ave	SEM	p-value
0	6.47	6.45	6.46	0.01	-	7.27	7.30	7.29	0.02	-
1	7.56	7.44	7.50	0.06	0.993	6.82	6.68	6.75	0.07	*0.001*
2	7.64	8.98	8.31	0.67	0.901	6.29	6.39	6.34	0.05	*0.001*
3	9.20	7.54	8.37	0.83	0.888	6.21	6.21	6.21	0.00	*0.001*
4	9.63	7.58	8.61	1.03	0.830	6.00	5.99	6.00	0.01	*0.001*
8	9.53	11.51	10.52	0.99	0.293	5.16	5.13	5.15	0.02	*0.001*
12	16.36	11.36	13.86	2.50	*0.027*	5.05	5.08	5.07	0.02	*0.001*

Table 8 – Three month stability testing of span and pH of liposomes at 37°C.

Week	Span					pH				
	1	2	Ave	SEM	p-value	1	2	Ave	SEM	p-value
0	6.47	6.45	6.46	0.01	-	7.19	7.15	7.17	0.02	-
1	7.60	7028	7.44	0.16	0.548	7.06	7.13	7.01	0.04	0.622
2	8.65	8.09	8.37	0.28	1.000	6.61	6.64	6.63	0.02	*0.001*
3	10.23	9.68	9.96	0.28	1.000	6.34	6.26	6.30	0.04	*0.001*
4	10.90	9.22	10.06	0.84	1.000	6.12	6.09	6.11	0.02	*0.001*
8	12.83	12.52	12.67	0.16	1.000	5.39	5.45	5.42	0.03	*0.001*
12	16.82	16.01	16.41	0.41	1.000	5.27	5.36	5.32	0.05	*0.001*

Table 9 – Three month stability testing of span, pH and entrapment efficacy (%EE) of Pheroid™ vesicles with mefloquine at 5°C.

Week	Span					pH					Entrapment Efficacy				
	1	2	Ave	SEM	p-value	1	2	Ave	SEM	p-value	1	2	Ave	SEM	p-value
0	3.00	2.98	2.99	0.01	-	4.55	4.56	4.56	0.01	-	58.73	58.83	58.78	0.05	-
1	2.95	3.00	2.98	0.03	0.993	4.11	3.87	3.99	0.12	*0.002*	48.59	51.53	50.06	1.47	0.948
2	2.91	2.93	2.92	0.01	0.165	3.96	3.86	3.91	0.05	*0.001*	56.02	82.21	69.12	13.10	0.896
3	2.96	2.91	2.93	0.03	0.344	3.69	3.59	3.64	0.05	*0.001*	44.10	48.85	46.48	2.38	0.807
4	2.91	2.87	2.89	0.02	*0.037*	3.51	3.49	3.50	0.01	*0.001*	53.74	64.66	59.20	5.46	1.000
8	3.08	3.10	3.09	0.01	*0.037*	3.16	3.08	3.12	0.04	*0.001*	58.03	60.38	59.21	1.18	1.000
12	3.09	3.09	3.09	0.00	*0.037*	3.02	2.99	3.01	0.02	*0.001*	81.14	63.66	72.4	8.74	0.737

Table 10 – Three month stability testing of span, pH and entrapment efficacy (%EE) of Pheroid™ vesicles with mefloquine at 25°C.

Week	Span					pH					Entrapment Efficacy				
	1	2	Ave	SEM	p-value	1	2	Ave	SEM	p-value	1	2	Ave	SEM	p-value
0	3.00	2.98	2.99	0.01	-	4.38	4.34	4.36	0.02	-	58.83	58.93	58.88	0.05	-
1	2.99	2.93	2.96	0.02	0.985	3.59	3.45	3.52	0.07	*0.002*	49.73	43.83	46.78	2.95	1.000
2	2.98	2.85	2.92	0.07	0.561	3.22	3.14	3.18	0.04	*0.001*	50.19	48.52	49.36	0.84	1.000
3	2.93	2.91	2.92	0.01	0.624	3.01	2.93	2.97	0.04	*0.001*	48.05	38.27	43.16	4.89	1.000
4	2.91	2.87	2.89	0.02	0.297	2.91	2.84	2.88	0.04	*0.001*	56.56	56.9	56.73	0.17	1.000
8	3.02	3.03	3.02	0.00	0.968	2.58	2.54	2.56	0.02	*0.001*	50.93	53.07	52.00	1.07	0.549
12	2.97	2.96	2.97	0.00	0.994	2.51	2.49	2.50	0.01	*0.001*	68.75	68.68	68.72	0.04	1.000

Table 11 – Three month stability testing of span, pH and entrapment efficacy (%EE) of Pheroid™ vesicles with mefloquine at 30°C.

Week	Span					pH					Entrapment Efficacy				
	1	2	Ave	SEM	p-value	1	2	Ave	SEM	p-value	1	2	Ave	SEM	p-value
0	3.00	2.98	2.99	0.01	-	4.12	4.14	4.13	0.01	-	58.83	58.89	58.86	0.03	-
1	2.58	2.59	2.58	0.00	*0.001*	3.33	3.22	3.28	0.06	*0.001*	48.18	47.58	47.88	0.30	0.436
2	2.97	2.91	2.94	0.03	0.499	3.02	2.98	3.00	0.02	*0.001*	51.00	46.51	48.76	2.25	0.514
3	2.89	2.91	2.90	0.01	0.082	2.84	2.82	2.83	0.01	*0.001*	43.36	46.98	45.17	1.81	0.245
4	2.91	2.86	2.88	0.03	*0.041*	2.76	2.74	2.75	0.01	*0.001*	57.36	67.41	62.39	5.03	0.990
8	2.89	2.93	2.91	0.02	0.131	2.51	2.5	2.51	0.01	*0.001*	48.72	63.26	55.99	7.27	0.997
12	2.81	2.83	2.82	0.01	*0.003*	2.47	2.46	2.57	0.01	*0.001*	64.53	70.36	67.45	2.92	0.663

Table 12 – Three month stability testing of span, pH and entrapment efficacy (%EE) of Pheroid™ vesicles with mefloquine at 37°C.

Week	Span					pH					Entrapment Efficacy				
	1	2	Ave	SEM	p-value	1	2	Ave	SEM	p-value	1	2	Ave	SEM	p-value
0	3.00	2.98	2.99	0.01	-	4.10	4.15	4.13	0.03	-	58.83	58.90	58.87	0.04	-
1	2.57	2.53	2.55	0.02	*0.002*	3.16	3.11	3.14	0.03	*0.001*	49.93	51.00	50.47	0.54	0.425
2	2.95	2.94	2.94	0.01	0.984	2.85	2.84	2.85	0.01	*0.001*	54.08	52.81	53.45	0.64	0.800
3	2.88	2.87	2.88	0.00	0.536	2.78	2.77	2.78	0.01	*0.001*	31.57	41.15	36.36	4.79	*0.008*
4	2.85	2.81	2.83	0.02	0.242	2.69	2.69	2.69	0.00	*0.001*	48.05	42.89	45.47	2.58	0.098
8	2.80	2.74	2.77	0.03	0.074	2.51	2.48	2.50	0.02	*0.001*	43.70	52.94	48.32	4.62	0.232
12	2.50	2.29	2.40	0.11	0.001	2.45	2.45	2.45	0.00	*0.001*	68.35	65.06	65.06	1.65	0.490

Table 13 – Three month stability testing of span, pH and entrapment efficacy (%EE) of liposomes with mefloquine at 5°C.

Week	Span					pH					Entrapment Efficacy				
	1	2	Ave	SEM	p-value	1	2	Ave	SEM	p-value	1	2	Ave	SEM	p-value
0	5.19	5.25	5.22	0.03	-	7.19	7.13	7.16	0.03	-	62.99	63.00	63.00	0.01	-
1	4.42	4.27	4.34	0.08	0.709	7.08	7.08	7.08	0.00	0.063	37.84	37.323	37.58	0.26	*0.001*
2	5.02	5.44	5.25	0.21	1.000	7.23	7.21	7.22	0.01	0.194	39.81	39.95	39.88	0.07	*0.001*
3	5.05	6.05	5.55	0.50	0.995	7.20	7.19	7.20	0.01	0.662	19.52	27.86	26.69	4.17	*0.001*
4	5.69	6.53	6.11	0.42	0.695	7.21	7.18	7.20	0.02	0.662	36.26	41.09	38.68	2.42	*0.001*
8	7.48	5.95	6.71	0.77	0.234	7.23	7.20	7.22	0.02	0.256	44.37	40.68	42.53	1.85	*0.002*
12	6.74	6.35	6.55	0.19	0.331	7.16	7.18	7.17	0.01	0.999	53.94	52.81	53.38	0.57	0.091

Table 14 – Three month stability testing of span, pH and entrapment efficacy (%EE) of liposomes with mefloquine at 25°C.

Week	Span					pH					Entrapment Efficacy				
	1	2	Ave	SEM	p-value	1	2	Ave	SEM	p-value	1	2	Ave	SEM	p-value
0	5.19	5.25	5.22	0.03	-	7.00	7.06	7.03	0.03	-	62.93	62.99	62.96	0.03	-
1	6.22	6.81	6.51	0.30	0.994	7.08	7.03	7.06	0.03	1.000	34.99	40.15	37.57	2.58	*0.019*
2	6.15	5.73	5.94	0.21	1.000	7.01	7.01	7.01	0.00	1.000	55.49	40.75	48.12	7.37	0.193
3	5.37	5.33	5.35	0.02	1.000	6.90	6.92	6.91	0.01	1.000	34.25	33.65	33.95	0.30	*0.009*
4	6.42	9.48	7.95	1.53	0.829	6.84	5.83	6.34	0.51	0.440	54.08	46.24	50.16	3.92	0.303
8	9.53	2.76	6.15	3.39	0.999	4.77	4.16	4.47	0.31	*0.001*	73.37	82.01	77.69	4.32	0.198
12	9.03	6.80	9.71	1.11	0.836	4.37	4.00	4.19	0.19	*0.001*	77.45	75.38	76.12	1.04	0.263

Table 15 – Three month stability testing of span, pH and entrapment efficacy (%EE) liposomes with mefloquine at 30°C.

Week	Span					pH					Entrapment Efficacy				
	1	2	Ave	SEM	p-value	1	2	Ave	SEM	p-value	1	2	Ave	SEM	p-value
0	5.19	5.25	5.22	0.03	-	6.99	6.97	6.98	0.01	-	62.98	62.80	62.89	0.09	-
1	5.66	5.83	5.75	0.09	1.000	6.77	6.79	6.78	0.01	0.554	54.08	56.76	55.42	1.34	0.409
2	5.15	6.12	5.63	0.48	1.000	6.84	6.84	6.84	0.00	0.833	36.53	41.82	39.18	2.65	*0.002*
3	5.33	6.08	5.71	0.37	1.000	6.25	6.25	6.25	0.00	*0.003*	45.44	55.28	50.36	4.92	0.074
4	11.02	16.27	13.64	2.63	0.172	4.93	5.20	5.07	0.14	*0.001*	61.45	61.61	61.53	0.08	0.999
8	4.31	11.65	7.98	3.67	0.948	4.38	4.54	4.46	0.08	*0.001*	75.98	80.0	77.99	2.01	*0.031*
12	6.33	12.05	9.19	2.86	0.797	4.20	4.45	4.33	0.13	*0.001*	71.02	75.25	73.14	2.12	0.163

Table 16 – Three month stability testing of span, pH and entrapment efficacy (%EE) of liposomes with mefloquine at 37°C.

Week	Span					pH					Entrapment Efficacy				
	1	2	Ave	SEM	p-value	1	2	Ave	SEM	p-value	1	2	Ave	SEM	p-value
0	5.19	5.25	5.22	0.03	-	7.01	7.09	7.05	0.04	-	62.90	62.99	62.95	0.05	-
1	5.90	6.97	6.43	0.54	0.978	6.69	6.76	6.73	0.04	*0.015*	45.51	45.44	45.48	0.04	0.060
2	5.38	5.28	5.33	0.05	1.000	6.68	6.61	6.65	0.04	*0.004*	53.54	42.63	48.09	5.46	0.118
3	5.72	6.44	6.08	0.36	0.996	6.02	5.86	5.94	0.08	*0.001*	62.18	52.54	57.36	4.82	0.868
4	7.77	13.26	10.51	2.74	0.090	4.99	4.91	4.95	0.04	*0.001*	64.93	66.54	65.74	0.81	0.994
8	6.64	7.56	6.80	0.76	0.860	4.46	4.40	4.43	0.03	*0.001*	67.74	73.17	70.46	2.72	0.669
12	4.49	4.49	4.49	0.00	0.998	4.39	4.31	4.35	0.04	*0.001*	77.72	84.82	81.27	3.55	0.048

Table 17 - Correlation between different characteristics of Pheroid™ vesicles.

	5 °C	25 °C	30 °C	37 °C
Span - pH	r = -0.0441 p = 0.881	r = -0.1467 p = 0.617	r = 0.2524 p = 0.384	r = 0.0325 p =0.912
Span - Degradation	r = -0.3496 p = 0.220	r = -0.2391 p = 0.410	r = -0.2839 p = 0.325	r = -0.2328 p = 0.423
pH – Degradation	r = -0.0763 p = 0.795	r = -0.117 p = 0.704	r = 0.0880 p = 0.765	r = 0.0909 p = 0.757
Time – Span	r = 0.2346 p = 0.419	r = 0.0815 p = 0.782	r = -0.2552 p = 0.379	r = 0.0059 p = 0.984
Time – pH	r = -0.0398 p = 0.893	r = -0.9671 *p = 0.000*	r = -0.9795 p = 0.000	r = -0.9359 *p = 0.000*
Time - Degradation	r = -0.1326 p = 0.651	r = 0.1757 p = 0.548	r = 0.0764 p = 0.795	r = -0.1048 p = 0.721

Table 18 - Correlation between different characteristics of liposomes.

	5 °C	25 °C	30 °C	37 °C
Span - pH	r = 0.149 p = 0.611	r = -0.4712 p = 0.089	r = -0.8060 *p = 0.000*	r = 0.3387 p = 0.236
Span - Degradation	r = 0.1267 p = 0.666	r = 0.3683 p = 0.195	r = -0.0384 p = 0.896	r = 0.1891 p = 0.517
pH – Degradation	r = -0.1840 p = 0.529	r = -0.433 p = 0.883	r = -0.1112 p = 0.705	r = 0.0248 p = 0.933
Time – Span	r = 0.5638 *p = 0.036*	r = 0.3535 p = 0.215	r = 0.8754 *p = 0.000*	r = -0.2289 p = 0.431
Time – pH	r = -0.3836 p = 0.176	r = -0.9684 *p = 0.000*	r = -0.9396 *p = 0.000*	r = -0.9441 *p = 0.000*
Time - Degradation	r = -0.2568 p = 0.375	r = -0.0579 p = 0.844	r = -0.0669 p = 0.820	r = -0.1336 p = 0.649

Table 19 - Correlation between different characteristics of Pheroid™ vesicles with mefloquine.

	5 °C	25 °C	30 °C	37 °C
Span - pH	r = -0.4815 p = 0.081	r = 0.1153 p = 0.695	r = 0.1195 p = 0.684	r = 0.4616 p = 0.156
Span - %EE	r = 0.2610 p = 0.367	r = 0.3612 p = 0.204	r = 0.1944 p = 0.506	r = -0.4001 p = 0.156
pH – %EE	r = -0.2737 p = 0.344	r = -0.2595 p = 0.370	r = -0.1569 p = 0.592	r = 0.1769 p = 0.545
Time – Span	r = 0.6949 *p = 0.006*	r = 0.2258 p = 0.438	r = -0.0052 p = 0.986	r = -0.6446 *p = 0.013*
Time – pH	r = -0.9046 *p = 0.000*	r = -0.8098 *p = 0.000*	r = -0.7762 *p = 0.001*	r = -0.7236 *p = 0.003*
Time - %EE	r = 0.4292 p = 0.126	r = 0.2609 p = 0.368	r = 0.5580 *p = 0.038*	r = 0.3611 p = 0.205

Table 20 - Correlation between different characteristics of liposomes with mefloquine.

	5 °C	25 °C	30 °C	37 °C
Span - pH	r = 0.5418 *p = 0.045*	r = -0.2606 p = 0.368	r = -0.5246 p = 0.054	r = -0.2648 p = 0.360
Span - %EE	r = 0.1845 p = 0.528	r = 0.0752 p = 0.7980	r = 0.3763 p = 0.185	r = 0.0071 p = 0.981
pH – %EE	r = -0.1307 p = 0.656	r = -0.7944 *p = 0.001*	r = -0.7520 *p = 0.002*	r = -0.7736 *p = 0.001*
Time – Span	r = 0.7440 *p = 0.002*	r = 0.3491 p = 0.221	r = 0.3654 p = 0.199	r = -0.0933 p = 0.751
Time – pH	r = 0.2725 p = 0.346	r = -0.9405 *p = 0.000*	r = -0.9147 *p = 0.000*	r = -0.9066 *p = 0.000*
Time - %EE	r = 0.1341 p = 0.648	r = 0.6858 *p = 0.007*	r = 0.6652 *p = 0.009*	r = 0.7998 *p = 0.001*

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Table 21 - Span and entrapment efficacy of Pheroid™ vesicles loaded with mefloquine during manufacturing.

Day	Span					Entrapment Efficacy				
	1	2	Ave	SEM	p-value	1	2	Ave	SEM	p-value
1	0.85	1.27	1.06	0.21	-	51.67	45.64	48.65	3.01	-
2	2.94	2.97	2.96	0.01	*0.0003*	25.75	21.19	23.47	2.28	*0.0092*
3	2.74	2.97	2.86	0.12	*0.0004*	34.72	47.52	41.12	6.40	0.9488
4	2.82	2.91	2.87	0.05	*0.0004*	40.01	37.94	38.98	1.04	0.7920
5	2.92	4.10	3.51	0.59	*0.0002*	40.35	46.44	43.40	3.05	0.9970
6	2.99	2.95	2.97	0.02	*0.0003*	43.90	44.70	44.30	0.40	0.9995
7	2.65	2.60	2.62	0.02	*0.0012*	52.00	33.72	42.86	9.14	0.9930
8	3.03	3.02	3.02	0.01	*0.0002*	38.47	38.67	38.57	0.10	0.7511
9	3.20	3.01	3.11	0.09	*0.0002*	43.43	46.24	44.84	1.41	0.9999
10	1.59	1.86	1.72	0.13	0.3813	36.66	42.43	39.55	2.88	0.8448
11	2.97	3.01	2.99	0.02	*0.0003*	52.67	60.24	56.46	3.78	0.9358
12	2.93	2.97	2.95	0.02	*0.0003*	53.21	49.19	51.20	2.01	1.0000
13	3.02	2.95	2.99	0.04	*0.0003*	53.41	51.20	52.30	1.11	0.9999
14	2.95	2.95	2.95	0.00	*0.0003*	58.10	58.20	58.30	0.20	0.7953

Table 22 - Span and entrapment efficacy of Pheroid™ vesicles loaded with mefloquine after manufacturing.

Day	Span					Entrapment Efficacy				
	1	2	Ave	SEM	p-value	1	2	Ave	SEM	p-value
1	1.89	1.79	1.84	0.05	-	62.45	69.55	66.00	3.55	-
2	2.62	2.70	2.66	0.04	0.8122	51.00	50.86	50.93	0.07	0.9222
3	4.48	2.59	3.54	0.94	0.0591	47.92	56.16	52.04	4.12	0.9522
4	5.15	5.05	5.10	0.05	*0.0003*	54.88	57.16	56.02	1.14	0.9967
5	2.04	1.98	2.01	0.03	1.0000	48.39	49.59	48.99	0.60	0.8468
6	3.23	2.73	2.98	0..25	0.4125	48.25	61.65	54.95	6.70	0.9919
7	3.96	3.68	3.82	0.14	*0.0196*	43.03	53.21	48.12	5.09	0.8044
8	2.07	2.07	2.07	0.00	1.0000	52.00	52.00	52.00	0.00	0.9514
9	3.02	2.78	2.90	0.12	0.5090	54.68	62.65	58.67	3.99	0.9998
10	2.19	2.28	2.23	0.05	0.9993	59.04	55.55	57.29	1.74	0.9991
11	2.72	2.70	2.71	0.01	0.7533	22.46	63.66	43.06	20.60	0.5092
12	2.97	2.08	2.52	0.45	0.9294	51.87	66.27	59.07	7.20	0.9999
13	2.06	2.79	2.42	0.36	0.9760	63.39	63.79	63.59	0.20	1.0000
14	2.76	2.74	2.75	0.01	0.6999	54.82	66.00	60.41	5.59	1.0000

Annexure C

Contains the certificate of analysis of mefloquine hydrochloride

Mod.05/01-8 REV.2 del 22/04/2004

Sifavitor S.p.A.
Via Livelli 1 - 26852 Casaletto Lodigiano Fraz. Mairano (ITALY)
Tel. 0371-73991 - Fax 0371-73108 - e-mail: sifavitor@mediolast.com

CERTIFICATE OF ANALYSIS

MEFLOQUINE HYDROCHLORIDE | Revision: 24/02/2005 | Page 1 of 1

Analysis Number 60604
Analysis Date 03/04/2006
Batch: 8700/03/06
Kg: 232.00
Formula: $C_{17}H_{16}F_6N_2O \cdot HCl$
M.W.: 414.80

Manufacturing Date 17/03/2006
Retest Date 03/2009
Complies with: Ph.Eur.-BP - USP

TEST	RESULTS	SPECIFICATIONS
DESCRIPTION Aspetto	*complies*	white or slightly yellow crystalline powder
IDENTIFICATION Identificazione	*complies* *complies*	(1): IR spectrum (2): chlorides test
APPEARANCE OF SOLUTION Aspetto della soluzione	*complies*	a 5% solution in methanol is clear and not more intensely coloured than reference solution BY7
OPTICAL ROTATION Rotazione ottica	*- 0.02°*	-0.20° to +0.20°
WATER Acqua	*2.04%*	not more than 3.0%
SULPHATED ASH Ceneri solforiche	*0.00%*	not more than 0.1%
HEAVY METALS Metalli pesanti	*complies*	not more than 20 ppm
RELATED SUBSTANCES (HPLC) Impurezze organiche (HPLC)	*(1): < LOD* *(2): < LOD* *(3): 0.03%* *(4): RRT 0.91: 0.01%* *RRT 2.03: 0.01%* *(5): 0.05%*	(1) impurity A nmt 0.10% (2) impurity B nmt 0.10% (3) impurity C nmt 0.20% (4) each unknown impurity nmt 0.10% (5) sum of all impurities nmt 0.50%
ASSAY Titolo	*100.43%*	btw.99.0% and 101.0% with reference to the anhydrous, solvent-free substance (pot.)
RESIDUAL SOLVENTS Solventi residui	*29 ppm*	acetone nmt 200 ppm

REPRESENTED BY:
D P Fine Chemicals (Pty) Limited
P O Box 708
RIVONIA 2128
Johannesburg
South Africa

10 YEARS
1995 2005

03/04/06	03/04/06	03.04.2006
Issue: QUALITY CONTROL	Control: QUALITY ASSURANCE	Approval: PLANT MANAGER

Annexure D

Contains the Ethics application for cultivation of *Plasmodium* falciparum parasites in human blood (NWU-0008-08-S5) and for evaluation of neurotoxicity (NUW-00025-09-S5)

NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT
POTCHEFSTROOM CAMPUS

Private Bag X6001, Potchefstroom
South Africa 2520

Tel: (018) 299-1111/2222
Web: http://www.nwu.ac.za

UNIT FOR DRUG RESEARCH AND DEVELOPMENT
Tel: (018) 018 299-2274
Fax (018) 018 293-5219
EMail jeanetta.duplessis@nwu.ac.za

Research Ethics Committee
North-West University
Potchefstroom Campus
Box116

15 April 2008

Dear Ms Botha

FINAL RESPONSE: ETHICS APPLICATION: NWU-0008-08-S5

The abovementioned application has reference.

We have received satisfacory answers to all the questions posed by the Ethics panel and has therefore found the ethical aspects to be in order.

PROF. J. DU PLESSIS
DIRECTOR

NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT
POTCHEFSTROOM CAMPUS

Private Bag X6001, Potchefstroom
South Africa 2520

Tel: (018) 299-1111/2222
Web: http://www.nwu.ac.za

UNIT FOR DRUG RESEARCH AND DEVELOPMENT
Tel: (018) 018 299-2274
Fax (018) 018 293-5219
EMail jeanetta.duplessis@nwu.ac.za

Research Ethics Committee
North-West University
Potchefstroom Campus
Box116

26 June 2009

Dear Mrs Halgryn

ETHICS APPLICATION: NWU-00025-09-S5

TOXICITY OF ANTI-MALARIAL DRUGS IN COMBINATION WITH PHEROID™ TECHNOLOGY

The above mentioned ethics application has been reviewed and the panel would like to remark the following:

1. Artemesinin and mefloquine are both scheduled substances in terms of the Medicines Control Act (Act 101 of 1965). The applicant shall indicate that the necessary permit in terms of the above mentioned act shall be obtained to hold and dispense the scheduled substances.
2. The applicant shall provide written evidence that Prof A.F. Kotze accepts his role as Responsible Pharmacist.
3. The names and contact details of the Professional Supervisors (Sec. 8b), Bio-Safety Officer (Sec. 8c), Terrain/Facility Managers (Sec. 8d), and Statistical Consultation Service (Sec. 8e) shall be added to the application.

The application in general is in compliance with current and relevant ethical codes. We therefore approve ethics application **NWU-0025-09-S5** provided that attention is given to point 1-3 above.

Yours sincerely

PROFJ DU PLESSIS
DIRECTOR