Formulation, characterization and cellular toxicity of lipid based drug delivery systems for mefloquine

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For all who believed and prayed

Our hope in difficult times is not based
on positive thinking, wishful thinking
or natural optimism. It is a certainty based
on the truths that God is in complete control
of our universe and that He love us.

~ Rick Warren ~

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This thesis is more than just experiments in the laboratories and words on paper, is a character test. One I would have failed without the support of my family. Suzette, Danie, Hannes and Riaan, thank you.

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And lastly, I would like to acknowledge God that enabled me to complete this thesis.

Preface

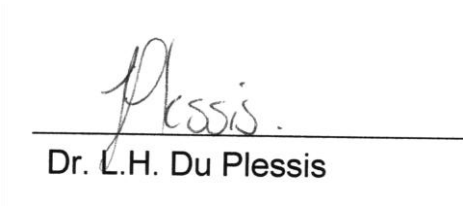
This thesis is submitted in fulfilment of the requirements of a Doctor of Philosophy in Pharmaceutics. This study forms part of the project T60042, Optimisation of drug delivery for tuberculosis, malaria and paediatric AIDS. This works was financially supported by the Innovation Fund. This thesis is submitted in an article format in accordance with the General Academic Rules (A.13.7.3) of the North-West University. Each chapter is written in accordance with specific guidelines as stipulated by the journals intended for publication. A short description about the specific guidelines are given before each chapter. Each chapter has its own list of abbreviations, table of contents, list of figures and list of tables were applicable. The outline of this thesis is as follows:

The contribution of each author is as follows:

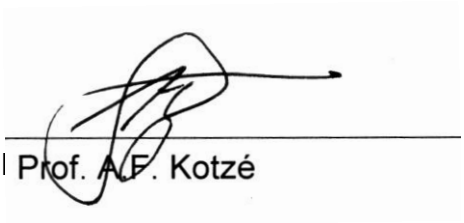
C. Slabbert	Responsible for the following under supervision of Dr. L.H. du Plessis and Prof. A.F. Kotzé: • Planning and design of study. • Experimental work. • Interpretation of results. • Writing of thesis and articles. Conference participation presented as posters, I was responsible for collecting and analysing data, interpreting results and writing of both abstracts. I compiled and was presenting author of the poster: Size determination of Pheroid™ formulations and liposomes.
Dr. L.H. du Plessis	As promotor of the candidate, I was responsible for: • Planning and design of study in collaboration with the candidate and co-promotor. • Assisted in interpretation of results. • Supervised writing of thesis and article. • Acts as corresponding author of articles.
Prof. A.F. Kotzé	Responsibility of co-promotor were as follows: • Assisted in the planning and design of the study in collaboration with the candidate and promoter. • Gave a critical review of the articles and thesis.
L. Nieuwoudt	Compiled and was presenting author of the poster: Entrapment ability of Pheroid™ formulations and liposomes.

Declarations

I hereby declare that I have approved the articles/thesis and that my role in the study as indicated above is representative of my actual contribution. I give permission as author or co-author for submission of articles.



Dr. L.H. Du Plessis



Prof. A.F. Kotzé

C. Slabbert

I hereby agree to the above mentioned author contribution and give permission for the use in this thesis.

L. Nieuwoudt

Summary

Malaria affects millions of people annually especially in third world countries. Increase in resistance and limited research being conducted adds to the global burden of malaria. Mefloquine, known for unwanted adverse reactions and neurotoxicity, is highly lipophilic and is still used as treatment and prophylaxis. Lipid drug delivery systems are commonly used to increase solubility and efficacy and decrease toxicity. The most generally used lipid drug delivery system is liposomes. The lipid bilayer structure varying in size from 25 nm to 100 μm can entrap both hydrophilic and lipophilic compounds. Similar in structure and size to liposomes, Pheroid™ technology consist of natural fatty acids and is also able to entrap lipophilic and hydrophilic compounds. The aim of this study was to formulate liposomes and Pheroid™ vesicles loaded with mefloquine and evaluate the physiochemical characteristic of the formulations followed by efficacy and toxicity studies.

Pheroid™ vesicles and liposomes with and without mefloquine were evaluated in size, morphology, pH and entrapment efficacy during three month accelerated stability testing. Optimization of size determination by flow cytometry lead to accurate determination of size for both Pheroid™ vesicles and liposomes. During the three months stability testing, Pheroid™ vesicles showed a small change in size from 3.07 ± 0.01 μm to approximately 3 μm for all three temperatures. Confocal laser scanning microscopic evaluation of the liposomes showed structures uniform in spherical shape and size. No difference in size or structure between the Pheroid™ vesicles with and without mefloquine were obtained. Significant increase ($p=0.027$) in size from 6.46 ± 0.01 μm to above 10 μm was observed for liposomes at all the temperatures. Clearly formed lipid bilayer structures were observed on micrographs. With the addition of mefloquine to the liposome formulation, a decrease in the amount of bilayer structures and an increase in oil droplets were found. Entrapment efficacy was determined by firstly separating the entrapped drug from the unentrapped drug utilizing a Sephadex®G50 mini column. This was followed by spectrophotometric evaluation by UV-spectrophotometry at 283 nm. Initial entrapment efficacy of both Pheroid™ vesicles and liposomes was above 60%. An increase in entrapment efficacy was observed for Pheroid™ vesicles. The addition of mefloquine to already formulated Pheroid™ vesicles illustrated entrapment efficacy of 60.14 ± 5.59% after 14 days. Formulations loaded with mefloquine resulted in lower pH values as well as a decrease in pH over time.

Optimization of efficacy studies utilizing propidium iodide was necessary due to the similarity in size and shape of the drug delivery systems to erythrocytes. A gating strategy was successfully implemented for the determination of the percentage parasitemia. Efficacy testing of mefloquine loaded in Pheroid™ vesicles and liposomes showed a 186% and 207% decrease in parasitemia levels compared to the control of mefloquine. Toxicity studies conducted include haemolysis and ROS (reactive oxygen species) analysis on erythrocytes as well as cell viability on mouse neuroblastoma cells. Pheroid™ vesicles with and without mefloquine resulted in a dose dependent increase in ROS and haemolysis over time. A dose dependent increase in ROS and haemolysis in both liposome formulations were observed, but to a lesser extent. Mefloquine proved to be neurotoxic with similar results obtained when mefloquine was entrapped in liposomes. Pheroid™ vesicles seem to have neuroprotective properties resulting in higher cell viability.

Mefloquine could be entrapped successfully in Pheroid™ vesicles and less in liposomes. Pheroid™ vesicles was more stable over a three months accelerated stability testing with more favourable characteristics. The increase in ROS levels of Pheroid™ vesicles could be responsible for the higher efficacy and haemolytic activity. DL-α-Tocopherol in Pheroid™ vesicles possibly acted as a pro-oxidant due to the presence of iron in the erythrocytes. DL-α-Tocopherol showed possible antioxidant properties in the neurotoxicity evaluation resulting in higher cell viability. Even though liposomes illustrated higher efficacy and little haemolysis and ROS production, no difference in neurotoxicity was observed together with unfavourable properties during stability testing makes this drug delivery system less favourable in comparison to Pheroid™ vesicles. Mefloquine was successfully incorporated into Pheroid™ vesicles resulted in high efficacy and showed possible neuroprotection and therefore makes it an ideal system for treatment of malaria.

Keywords:

- Pheroid™ Technology
- Liposomes
- Mefloquine
- Efficacy
- Neurotoxicity
- Haemolysis
- ROS analysis

Uittreksel

Formulering, karakterisering en sellulêre toksisiteit van lipied gebaseerde geneesmiddelafleweringsisteme vir meflokien

Malaria affekteer miljoene mense, veral in derde wêreldse lande op 'n jaarlikse basis. Faktore wat bydrae tot die globale druk van malaria is die verhoging in weerstand en beperkte navorsing wat in hierdie veld gedoen word. Die hoogs lipofiliese geneesmiddel, meflokien, is bekend vir sy ongewenste newe effekte en neurotoksisiteit, maar word nogsteeds in die behandeling en voorkoming van malaria gebruik. Lipied geneesmiddelafleweringsisteme word algemeen gebruik vir die verhoging in oplosbaarheid en verlaging in toksisiteit. Die mees algemeen bekende en gebruikte lipied afleweringsisteem is liposome. Die sferiese dubbelmembraan struktuur wat wissel in grootte van 25 nm tot 100 μm kan beide hidrofiliese en lipofiliese middels vasvang. Pheroid™ tegnologie wat bestaan uit natuurlike vetsure is eenders in struktuur en grootte wanneer vergelyk word met liposome. Pheroid™ tegnologie kan ook hidrofiliese en lipofiliese middels vasvang. Die doel van hierdie studie was om meflokien in beide liposome en Pheroid™ vesikels te formuleer en die fisiese-chemiese eienskappe, effektiwiteit en toksisiteit van dié formules te evalueer.

Grootte, morfologie, pH en geneesmiddel enkapsulering effektiwiteit van Pheroid™ vesikels en liposome met en sonder meflokien oor drie maande versnelde stabiliteit toetse was geëvalueer. Optimalisering van grootte bepaling deur middel van vloeisitometrie het gelei tot die akkurate bepaling van die grootte van Pheroid™ vesikels en liposome. Pheroid het 'n klein verandering in grootte van 3.07 ± 0.01 μm tot ongeveer 3 μm getoon by al die temperature gedurende die stabiliteit toetse. Konfokale laser skandeer mikroskopie observasie het sferiese strukture uniform in grootte getoon. Toevoeging van meflokien by die Pheroid™ vesikels tydens formulering het gelei tot geen verandering in grootte of struktuur nie. Liposome by al die temperature het 'n betekenisvolle vergroting ($p=0.027$) van 6.46 ± 0.01 μm tot bo 10 μm getoon. Duidelik gevormde dubbelmembraan strukture is waargeneem op die mikrograwe van die liposome. 'n Verlaging in die hoeveelheid dubbelmembraan strukture en 'n verhoging in olie druppels is waargeneem met die toevoeging van meflokien. Bepaling van die enkapsulering effektiwiteit was in twee stappe gedoen. Eerstens is die vry meflokien geskei van die vasgevangde meflokien deur gebruik te maak van 'n Sephadex®G50 mini kolom. Daarna is die vry meflokien spektrofotometries bepaal met 'n UV-spektrofotometer by 'n golflengte van 283 nm. Die

aanvangs enkapsulering effektiwiteit van beide Pheroid™ vesikels en liposome was bo 60%. Pheroid™ vesikels het op 'n verhoging in geneesmiddel inhoud gedui na drie maande. Die byvoeging van meflokien by klaar geformuleerde Pheroid™ vesikels het 'n 60.14 ± 5.59% enkapsulering effektiwiteit na 14 dae getoon. Formules met meflokien het laer pH waardes gehad sowel as 'n verlaging in pH oor tyd.

As gevolg van die ooreenkomste in grootte en struktuur tussen die afleweringsisteme en rooibloedselle, was optimalisering vir die bepaling van effektiwiteit met propidium iodied nodig. Die persentasie parasitemia is bepaal deur die implementering van 'n data genering strategie. 'n Verlaging van 186% vir Pheroid™ vesikels en 207% vir liposome ten opsigte van die kontrole was waargeneem tydens die effektiwiteit studies. Toksisiteit studies op rooibloedselle het hemolise en reaktiewe suurstof spesies (RSS) ingesluit en neurotoksisiteit is bepaal op muis neuroblastoma selle. 'n Dosis afhanklike verhoging oor tyd in RSS en hemolise is waargeneem vir Pheroid™ vesikels met en sonder meflokien. Liposome het ook 'n dosis afhanklike verhoging in RSS en hemolise getoon, maar tot 'n mindere mate. Neurotoksisiteit van meflokien was bewys in die kontrole en wanneer geënkapsuleer was in liposome. Pheroid™ vesikels het 'n moontlike neurobeskermende eienskap wat lei tot verhoogde sel lewensvatbaarheid.

Meflokien kon suksesvol in Pheroid™ vesikels geënkapsuleer word maar tot 'n mindere mate in liposome. Pheroid™ vesikels was meer stabiel tydens die drie maande versnelde stabiliteit toetse met gunstige eienskappe. Die verhoogde RSS vlakke wat waargeneem is by Pheroid™ kan verantwoordelik wees vir die hoër effektiwiteit en hemolise. DL-α-tokoferol in Pheroid™ vesikels tree op as pro-oksidant in die teenwoordigheid van yster in rooibloedselle. Die verhoogde sel lewensvatbaarheid wat waargeneem is by die neurotoksisiteit evaluering van Pheroid™ vesikels kan toegeskryf word aan die antioksidant eienskap van DL-α-tokoferol. Liposomes het verhoogde effektiwiteit met min hemolise en RSS produksie. Die ongewenste stabiliteitsdata en die feit dat geen neurobeskerming waargeneem was by liposome nie, maak hierdie geneesmiddelaflewering sisteem minder gunstig as Pheroid™ vesikels. Meflokien was suksesvol in Pheroid™ vesikels geënkapsuleer wat gelei het tot hoë effektiwiteit met moontlike neurobeskerming wat hierdie sisteem ideaal maak vir die behandeling van malaria.

Sleutelwoorde:

- Pheroid™ tegnologie
- Liposome
- Meflokien
- Effektiwiteit
- Neurotoksisiteit
- Hemolise
- RSS analise