Preparation, stability and *in vitro* evaluation of liposomes containing amodiaquine

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(B.Pharm)

Dissertation submitted in fulfilment of the requirements for the degree

MAGISTER SCIENTIAE (PHARMACEUTICS)

at the

POTCHEFSTROOM CAMPUS OF THE NORTH-WEST UNIVERSITY

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November 2010

Potchefstroom

“Do not pray for easy lives.
Pray to be stronger men!
Do not pray for tasks equal
to your powers. Pray for
powers equal to your tasks.
Then the doing of your
work shall be no miracle,
but you shall be a miracle”
- Philip Brooks

“The most exciting phrase
to hear in science, the one
that heralds new
discoveries, is not ‘Eureka!’
but ‘That’s funny...’”
- Isaac Asimov

Acknowledgements

I would like to start off by thanking and praising our Heavenly Father for the opportunities, the abilities and the blessings that I have received thus far in my life.

I would like to thank my parents, **Jacques** and **Sarita Scholtz** for giving me life and raising me to be the person I am today. I would also like to thank my sister, **Nadia Scholtz**. Thank you all for paving the way towards my academic success. Nothing could replace your love, support and care.

I would like to thank Dr. **Lissinda du Plessis**, my supervisor for her help, guidance and patience with me and my strange ways. Your help and guidance was paramount to the successful completion of my study.

Prof. **Awie Kotzé** my co-supervisor for always having an open door policy, and making the time to listen and help.

The **Innovation fund** for their monetary support.

Stephnie Nieuwoudt, thank you for walking this road with me and always being there to help and to encourage me. The blood sweat and tears shed in this time will bear fruit for us both.

Prof. **Wilna Liebenberg** for the use of her laboratories and equipment. I appreciate it very much.

Prof. **Lesley Greyvenstein** for the language editing.

I would like to thank all my friends **Christo, Jeanine, Ruan, Nicolene, Theunis, Chucky, HeLska, Cerenus, Michael, Geodelle, Jandré, Michelle** and **Lizl** for your unfailing support and friendship during the course of my study. You guys and girls mean the world to me.

To my family (especially the **Bothma**'s), your support in the tough times really helped me through.

To all my colleagues at the Department of Pharmaceutics, thank you for the fun times, chatting and joking in the office. Wish you all the best and success for your future.

Special thanks go out to **Chrizaan Slabbert, Righard Lemmer** and **Herman van der Watt**. The help and guidance you each gave me in each separate field where you specialise was just amazing. Thank you for your time and help that you offered so willingly. Thank you all very much. People like you make this world a fantastic place and I can't wait to take on the world with you all by my side.

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List of Abbreviations

AQ: Amodiaquine

CDC: Centre for Disease Control

CL: Conventional liposomes

DCFH-DA: 2',7'-dichlorofluorescein diacetate

DOH: Department of Health (South Africa)

EDL: Essential Drug List

FACS: Fluorescence Activated Cell Sorter

FP: Ferriprotoporphyrin IX

Fluorescein-DHPE: N-(fluorescein-5-thiocarboyl)-1,2-diheade-canoyl-sn-glycero-3-phosphoethanolamine

FSC: Forward scatter

Hb: Haemoglobin

iRBC: *Plasmodium* infected Red blood cells

LCL: Long Circulating Liposomes

LMWA: Low molecular weight antioxidants

LUV: Large Unilamellar Vesicles

MLV: Multilamellar Vesicles

NIAID: National Institute of Allergy and Infectious diseases

NOS: Reactive nitrogen species

OLV: Oligolamellar Vesicles

PBS: Phosphate buffer solution

PC: Phosphatidyl choline

RBC: Red blood cells or erythrocytes

ROS: Reactive oxidative species

RPMI: Roswell Park Memorial Institute (refers to the buffer)

SSC: Side scatter

SUV: Small Unilamellar Vesicles

T_C: Phase transition temperature

UV: Ultra violet

WHO: World Health Organization

Abstract

Title: Preparation, stability and *in vitro* evaluation of liposomes containing amodiaquine.

Keywords: Malaria, liposomes, amodiaquine, Plasmodium falciparum, stability, toxicity, entrapment efficacy, size determination, reactive oxygen species, lipid peroxidation.

Malaria is a curable disease that claims nearly one million lives each year. Problems with the treatment of malaria arise as resistance spreads and new treatment options are becoming less effective. The need for new treatments are of the utmost importance. Liposomes combined with antimalarials are a new avenue for research as liposomes can increase the efficacy of drugs against pathogens, as well as decreasing toxicity. Amodiaquine is a drug with known toxicity issues, but has proven to be effective and is, therefore, a prime candidate to be incorporated into the liposomal drug delivery system.

The aim of this study was to prepare, characterize and evaluate the toxicity of the liposomes with incorporated amodiaquine. The solubility of amodiaquine was determined and liposomes formulated with, and without, amodiaquine entrapped. Accelerated stability studies (at 5 ºC, 25 ºC with relative humidity of 60% and 40 ºC with a relative humidity of 40%) were conducted during which the size, pH, morphology and the entrapment efficacy was determined. The toxicity was determined *in vitro* by analysing the levels of reactive oxidative species and lipid peroxidation caused by the formulations to erythrocytes infected with *P. falciparum* as well as uninfected erythrocytes with flow cytometry.

The solubility study of amodiaquine in different pH buffers showed that amodiaquine was more soluble at lower pH values. Solubility in solution with pH 4.5 was 36.3359 ± 0.7904mg/ml when compared to the solubility at pH 6.8, which was 15.6052 ± 1.1126 mg/ml. A buffer with a pH of 6 was used to ensure adequate solubility and acceptable compatibility with cells. Liposomes with incorporated amodiaquine were formulated with entrapment efficacies starting at 29.038 ± 2.599% and increasing to 51.914 ± 1.683%. The accelerated stability studies showed the median sizes and span values remained constant for both liposome and amodiaquine incorporated liposomes at 5 ºC. The higher temperatures, i.e. 25 ºC and 40 ºC, displayed increases in the median size, and decreases in the span for both formulations. The conclusion can, therefore, be made that both liposome and amodiaquine incorporated liposomes are stable at lower temperatures. The entrapment efficacy increased from initial values to nearly 100% during the course of the stability study. This was attributed to amodiaquine precipitating from the solution. The pH values of the liposomes and amodiaquine incorporated liposomes remained

constant for each formulation; though the amodiaquine incorporated liposomes had a lower starting pH, the formulations are both thought to be stable in terms of the pH.

Toxicity studies revealed low levels of reactive oxygen species as well as low levels of lipid peroxidation for both liposome and amodiaquine incorporated liposomes, on both erythrocyte and *Plasmodium* infected erythrocytes. From the toxicity studies it can be concluded that liposomes and amodiaquine incorporated liposomes are not toxic to erythrocytes and infected erythrocytes.

It was concluded that liposomes incorporating amodiaquine could possibly be used as a treatment option for malaria.

Uittreksel

Titel: Die vervaardiging, stabiliteit en *in vitro* evaluering van amodiakien bevattende liposome

Sleutelwoorde: Malaria, liposome, amodiakien, *P. falciparum*, stabiliteit, toksisiteit, inkorporerings effektiwiteit, groottebepaling, reaktiewe suurstof spesies, lipied peroksidasie.

Malaria is ŉ geneesbare toestand wat meer as ŉ miljoen lewens elke jaar eis. Probleme met die behandeling van malaria duik op as gevolg van verspreidende weerstandbiedendheid van die parasiete teen huidige behandelings. Daarom is dit uiters belangrik om nuwe behandelings te ontwikkel. Die kombinasie van liposome met antimalaria middels is ŉ nuwe veld wat ondersoek kan word, omdat liposome die effektiwiteit teen verskeie patogene kan verbeter, sowel as om toksisiteit te verlaag. Probleme wat met amodiakien toksisiteit ondervind word, is welbekend, maar die middel beskik oor hoë effektiwiteit. Daarom is amodiakien ŉ geskikte middel om in ŉ afleweringssisteem ingesluit te word.

Die doel van die studie was om liposome en liposome met geïnkorporeerde amodiakien te vervaardig, te karakteriseer en die toksisiteit daarvan te evalueer. Die oplosbaarheid van amodiakien is bepaal en liposome berei, met en sonder die geneesmiddel daarin geïnkorporeer. Versnelde stabiliteitsstudies (in 5 ºC, 25 ºC met ŉ relatiewe humiditeit van 60% en 40 ºC met ŉ relatiewe humiditeit van 40%) was gedoen, waartydens die grootte, pH, morfologie en inkorporerings effektiwiteit bepaal is. Daarna is die toksisiteit *in vitro* bepaal deur die vlakke van reaktiewe suurstof spesies en vlakke van lipied peroksidase, wat veroorsaak is deur verskillende formulerings op *Plasmodium* geïnfekteerde rooibloedselle en on-geïnfekteerde rooibloedselle, deur middel van vloeisitometrie.

Die oplosbaarheid van amodiakien in verkillende pH buffers is bepaal. Die oplosbaarheid studies het getoon dat amodiakien meer oplosbaar is by laer pH waardes. Oplosbaarheid by pH 4.5 was 36.3359 ± 0.7904mg/ml, in vergelyking met 15.6052 ± 1.1126mg/ml by pH 6.8. ŉ Buffer met ŉ pH van 6 is dus gebruik, om te verseker dat die amodiakien voldoende sal oplos, sowel as om verenigbaarheid met die selkulture te verseker. Liposome met amodiakien geïnkorporeer, kon dus vervaardig word, met aanvanklike geneesmiddel inkorporering wat begin by 29.038 ± 2.599% en styg tot 51.914 ± 1.683%. Versnelde stabiliteitsstudies het getoon dat grootte, sowel as die deeltjie verspreiding relatief konstant gebly het vir beide die liposome en amodiakien geïnkorporeerde liposome by 5 ºC. Die hoër temperature, dit wil sê 25 ºC en 40 ºC, het ŉ verhoging in die grootte en ŉ afname in deeltjie verspreiding getoon. Hieruit kan afgelei word dat beide formulerings stabiel is by laer temperature. Die inkorporeringseffektiwiteit van die geneesmiddel het gestyg van die aanvanklike waardes tot byna 100% by al die

temperature gedurende die stabiliteitsondersoek. Dit kan toegeskryf word aan die presipitasie van amodiakien uit die oplossing. Die pH waardes van beide formulerings het konstant gebly, alhoewel die amodiakien liposome oor ’n laer aanvanklike pH geskik het. Beide formulerings is stabiel geag in terme van pH.

Toksisiteit studies het lae vlakke reaktiewe suurstof spesies, sowel as lae vlakke lipied peroksidase vir beide liposoom en amodiakien geïnkorporeerde liposoomformulerings op rooibloedselle en *Plasmodium* geïnfekteerde rooibloedselle getoon. Vanuit die toksisiteitbepaling kan afgelei word dat liposome en liposome met amodiakien geïnkorporeer, nie toksies vir rooibloedselle is nie.

Uit die resultate kan die afleiding gemaak word dat liposome waarin amodiakien geïnkorporeer is, ’n moontlike behandelings opsie vir malaria kan wees.

Introduction and aim of study

Worldwide, more than 1 million people die as a result of malaria. This serious disease affects the lives of more than 1.62 billion people that live in areas where malaria is endemic (WHO, 2009; CDC, 2010; Daily, 2006). Unfortunately, malaria is most wide-spread and out of control in developing countries that do not have sufficient infrastructure to handle a health crisis on such a large scale (WHO, 2009). This problem is further aggravated by the fact that malaria resistance is becoming an ever increasing and wide spread problem. This leads to inadequate treatment and treatment failures (Wongsrichanalai *et al.*, 2002). Even newly introduced treatments are not safe from the threat of treatment failure, as even the newly introduced artemisinin treatment alternatives have shown the first stage of treatment failures due to resistance (Dondorp *et al.*, 2009). Therefore, it is important to develop new treatment options and review treatment regimes.

For many years chloroquine has been the staple of malaria treatment, but chloroquine has come under fire as resistance started spreading and is now almost a global occurrence (Foley & Tilley, 1997). An alternative to chloroquine is amodiaquine, as cross-resistance to both amodiaquine and chloroquine is rare, and amodiaquine has increased efficacy even when chloroquine resistant malaria was tested (Foley & Tilley, 1997; Hawley *et al.*, 1996; Winstanley *et al.*, 1990). Amodiaquine may be an answer to many problems, but amodiaquine has an unfortunate stigma attached to it as certain severe side-effects, encountered in the 1980's, removed amodiaquine from wide-spread and prophylactic use. In 1996 the WHO reintroduced amodiaquine to the essential drug list, as extensive research showed that amodiaquine related serious side-effects are rare (Olliaro & Taylor, 2003). Unfortunately not much research has been done on amodiaquine as the use thereof has been limited (Winstanley *et al.*, 1990).

Problems in malaria treatment, such as resistant parasites and toxicity can in a large part be decreased and controlled if a drug delivery system is employed. A lipid based drug delivery system known as liposomes has proven itself to be useful in both these respects, as liposomes have in past studies, improved pharmacokinetics and bio-distribution, decreased toxicity and increased efficacy against a wide range of pathogens (Sharma & Sharma, 1997; Drulis-Kawa *et al.*, 2006). Unfortunately, as with most things in life, liposomes as a drug delivery system is not without its faults, and this needs to be closely examined as many different aspects, especially the physicochemical aspects of formulations need to be tested and examined before starting *in vivo* tests (New, 1990). It has been shown that drugs, including arthemether, chloroquine, primaquine and a whole host of others have been successfully incorporated into liposomes. The

formulations showed an increase in bioavailability, possibly overcoming resistance and often a decrease in toxicity (Qui *et al.*, 2008; Sharma & Sharma, 1997; Bayomi *et al.*, 1998).

The aims of this study were the preparation, characterisation and *in vitro* evaluation of liposomes containing amodiaquine. Therefore, in this study, a combination of amodiaquine and liposomes was prepared and tested to determine if a combination was possible and viable to formulate. Preliminary studies were done to determine if a possible combination is safe for use.

Therefore, the specific objectives of this study were:

1. Manufacturing liposomes according to the thin film hydration method.
2. Characterisation of liposomes according to morphology, size, pH and entrapment efficacy.
3. Manufacturing liposomes and incorporating amodiaquine.
4. Characterising amodiaquine entrapped liposomes according to size and entrapment efficacy.
5. Determining the stability of said formulations under high stress situations, such as accelerated stability testing.
6. To evaluate the possible toxicity of liposomes and liposomes incorporated with amodiaquine.

Chapters 1 to 3 consist of a literature study covering malaria, liposomes as a drug delivery system and the determination of the physicochemical properties of the formulations as well as the toxicity determinations. Chapter 4 consists of the experimental design, methods followed, the results and the discussions of said experiments. This study is unique as this author was unaware of any studies using a combination of liposomes and amodiaquine. This study will help determine if amodiaquine combined with liposomes is possible, viable and safe. If this is deemed to be the case, further studies may optimise this system, test its efficacy against different *Plasmodium* strains and may even move it to wide spread production and use.