Formulation and testing of novel pediatric antimalarial dosage form

and a pilot preclinical study in rabbits

Ellen Kalesi Gondwe Mhango

Thesis for the degree of Philosophiae Doctor

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Þróun og prófun lyfjaforms til meðhöndlunar á malaríu í börnum

og forklínískar rannsóknir í kanínum

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Ágrip

Mýrarkalda (malaría) er enn í dag stórt alþjóðlegt vandamál, sérstaklega í Afríku, sunnan Sahara, þar sem fjöldi barna deyja vegna sjúkdómsins. Á hverri mínútu deyr barn í Afríku vegna malaríu. Til að minnka líkur á lyfjaónæmum malaríustofnum hefur Alþjóða Heilbrigðismálastofnunin (WHO) farið fram á að nota skuli samsettar lyfjablöndur þar sem annað þeirra er úr flokki artemisína (ACT). WHO hefur lagt til að nota skuli samsetningu sem inniheldur lyfin artemeter (AR) og lumefantrín (LF), í ákveðnu hlutfalli, til meðhöndlun á malaríu af völdum sníkilsins *Plasmodium falciparum*.

Í dag eru blöndur af AR og LF eingöngu til sem föst lyfjaform (töflur oþh). Börn með alvarlega malaríu eru oftar en ekki meðvitundarlaus og eiga erfitt með að kyngja lyfjum til inntöku. Það er ekki auðvelt að útbúa fljótandi lyfjaform með þessum lyfjum, þar sem þau þurfa að vera á uppleystu formi, vegna þess hve torleyst þau eru.

Markmið þessa verkefnis er því að þróa fljótandi lyfjaform fyrir börn, sem inniheldur bæði lyfin í ákveðnu hlutfalli, ætlað til inngjafar um endaþarm; meta eðlisefnafræðilega þætti þessa lyfjaforms og framkvæma samanburðarrannsókn í kanínum.

Forrannsókn var framkvæmd til að meta hvaða leysar gætu leyst upp bæði lyfin. Síðan var ósamrýmanleikinn skoðaður milli AR og LF til að átta sig á hvers vegna LF félli út í völdum leysum. Í framhaldi af því voru þeir þættir fundnir sem skiptu máli fyrir leysanleikann og útfellingu LF í lausnum. Valið lyfjaform var síðan prófað í kanínum í samanburðarrannsókn.

Þegar fitusýrum var blandað við þetta lyfjaform, þá tókst að hindra útfellingar af LF, þannig að lyfin héldust í uppleystu formu. Forritið Design of Experiment nýttist vel til að finna þá þætti sem skiptu máli til að halda LF í upplausn. Forritið benti einnig á hvaða svigrúm væri til staðar án þess að útfelling myndi eiga sér stað og hvaða styrkir væru bestir til að fá fram endurtakanlegar niðurstður. Fitusýrurnar voru nauðsynlegar til að halda LF uppleystu, en þörf er á áframhaldandi rannsóknum til að skilja hvaða verkunarmátar bera ábyrgð á þessum áhrifum.

Bornar voru saman lyfin AR og LF í formi dreifu til inntöku og endaþarmsvökva, í heilbrigðum kanínum. Aðgengi LF reyndist marktækt hærra þegar lyfið var gefið í formi endaþarmsvökva, samanborið við sama skammt gefið um munn. Tíminn að hæsta blóðstyrk (t_{max}) var óvenju langur og þarfnast nánari rannsóknar, sérstaklega m.t.t. líffærafræðilegs og lífeðlisfræðilegs munar á kanínum og mönnum.

Það er nokkuð ljóst að það verður mikilvægt að aðlaga og minnka skammtinn af LF þegar lyfið verður gefið í formi endaþarmsvökva, til að ná sambærilegum blóðstyrk og

eftir inngjöf um munn. Jafnvel þótt þessi forklíníska rannsókn hafi verið gerð í litlu úrtaki, þá veita gögnin mikilvægar upplýsingar um hvernig hægt verði að bjóða upp á bráðameðferð við alvarlegri malaríu eða heilamalaríu í börnum. Næsta skref er að framkvæma klínískt próf með fleiri þátttakendum svo hægt verði að bjóða upp á neyðarlyf fyrir börn með alvarlega malaríu eða heilamalaríu.

Lykilorð: artemeter, lumefanrín, malaría, malaríulyf, leysanleiki, börn, skammtar, samanburður, lyfjahvarfafræði.

Abstract

Malaria remains a global challenge, especially in Sub-Saharan Africa where many children die due to this disease. It is reported that every minute a child dies due to malaria in Africa. To slow the development of resistance in drugs used to treat malaria, the World Health Organization (WHO) recommended use of artemisinin-based combination therapies for the treatment of malaria. Artemether (AR) and lumefantrine (LF) fixed dose combination is one of the artemisinin-based combination therapies (ACT) that was recommended by WHO for the treatment of uncomplicated *P. falciparum* malaria.

At present, fixed dose combination therapies containing AR – LF are available only as oral solid dosage forms commercially. However, children who have severe malaria and are unconscious or those with swallowing difficulties cannot take oral medications. Formulating parenteral dosage forms especially solutions, whereby both AR and LF need to be in dissolved state in the same formulation, is difficult due to the poor solubilities of these drugs.

Therefore, the aims of this project were to develop a pediatric liquid formulation containing a fixed dose combination of AR and LF for rectal delivery; to evaluate the physicochemical properties of the formulation and to conduct a bioequivalence study in rabbits.

A prefomulation study was conducted to screen potential solvents that could solubilize both drugs. An interaction study between AR and LF was conducted to find the cause of precipitation of LF in the chosen solvent. Further experiments were conducted to identify factors that were significant for the dissolution and precipitation time of LF. The potential formulation was tested in rabbits in a bioequivalence study.

Fatty acids were found to prevent precipitation when mixed into the lipophilic formulation, as LF became soluble. Design of Experiment helped in identifying factors that were significant for the dissolution and precipitation time of LF. It also aided in identifying the range or limits in which factors could be investigated to achieve optimal and reproducible working conditions. The inclusion of a fatty acid was necessary to keep LF in solution, but further work is needed to verify the mechanism of interaction behind LF precipitation.

AR-LF oral suspension and rectal enema were compared and successfully administered to healthy rabbits. The bioavailability of LF showed that a significantly higher bioavailability was found following rectal administration, compared with the oral route.

The t_{max}, however, was surprisingly long partly due to formulation excipients and the anatomical and physiological difference of the rectum of rabbits and humans.

In conclusion, the observed data suggest that a significant adjustment in the dose will be required when LF is administered via the rectal route, to receive comparable plasma levels as for healthy adults. Although the preclinical study had low power, due to the small sample size, the data provide important information for the next step in finding a method to provide a rescue treatment for children with severe or cerebral malaria. A clinical study with high power is planned as the next step in providing an effective rescue treatment for children suffering from severe or cerebral malaria.

Keywords: artemether, lumefantrine, malaria, antimalarial, solubility, pediatrics, dosage, bioequivalence, pharmacokinetics

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Contents

List of Abbreviations

ACN	Acetonitrile
ACTs	Artemisinin based combination therapies
AR	Artemether
ATSB	Attractive toxic sugar bait
APIs	Active pharmaceutical ingredients
AUC	Area under the plasma drug concentration time curve
B/P	Blood to plasma ratio
CARAMAL	Community access to rectal artesunate
$CDCl_3$	Deuterated chloroform
Cl	Clearance
C_{max}	Maximum concentration
CYP	Cytochrome
DAI	Dual active ingredient
DCA	Decanoic acid
DDT	Dichlorodiphenyltrichloroethane
DEET	Diethyltoluamide
DF	Degrees of freedom
DHA-PPQ	Dihydroartemisin - piperaquine
dL	Decilitre
ESI	Electrospray Ionization
F	Bioavailability
fu	Fraction unbound
g	Gram
GMEP	Global Malaria Education Campaign
GIT	Gastrointestinal tract
H	Hour
Hg	Mercury
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
HT	Hollow type
IGR	Insect growth regulator
IM	Intramuscular
IPTp	Intermittent preventive treatment of malaria in pregnant women
IPTi	Intermittent preventive treatment of malaria in infants
IRS	Indoor residual spraying

IS	Internal standard
ITNs	Insecticide treated nets
ITT	Incompatible insecticide technique
IU	International units
IV	Intravenous
K_a	Absorption rate constant
Kg	Kilogram
L	Litre
LF	Lumefantrine
LLINs	Long lasting insecticide nets
MCT	Medium chain triglyceride
MeOH	Methanol
mEq	Milliequivalent
Mg	Milligram
min	Minute
mL	Millilitre
mmoL	Millimoles
MMV	Medicines for Malaria Venture
MPAG	Malaria policy advisory group
mPEG	Methoxy polyethylene glycol
MLR	Multiple linear regression
m/z	Mass to charge ratio
N	Number of experiments (runs)
NE	No effect
Ng	Nanogram
NLC	Nanostructured lipid carriers
Nm	Nanometer
NMR	Nuclear Magnetic Resonance
NZW	New Zealand White
OA	Oleic acid
PDA	Photodiode array detector
Ph. Int.	International Pharmacopoeia
PMC	Perennial malaria chemoprevention
QC	Quality control
RAS	Rectal artesunate suppository
RBCs	Red blood cells
RPM	Revolutions per minute
SAGE	Strategic advisory group of experts
SP	Sulfadoxine pyrimethamine
RSD	Residual standard deviation
SDY	Standard deviation of the response

T_{max}	Time to reach maximum concentration
$t_{1/2}$	Half life
µL	Microlitre
µmoL	Micromole
ULV	Ultra-low volume
UPLC–MS/MS	Ultra performance liquid chromatography tandem mass spectrometry
UV/Visible	Ultraviolet visible spectroscopy
V_d	Volume of distribution
WHO	World Health Organization
VWD	Variable wavelength detector

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List of Original Papers

This thesis is based on the following original publications:

I. Ellen K.G. Mhango, Benjamin R. Sveinbjornsson, Bergthora S. Snorradottir & Sveinbjorn Gizurarson. Incompatibility of antimalarial drugs: challenges in formulating combination products for malaria, *Drug Delivery*, **31 (**1) 2299594 (p.1-10), 2024.

II. Mhango, E.K.G.; Snorradottir, B.S.; Kachingwe, B.H.K.; Katundu, K.G.H.; Gizurarson, S. Estimation of Pediatric Dosage of Antimalarial Drugs, Using Pharmacokinetic and Physiological Approach. *Pharmaceutics* **15**, 1076, 2023.

III. Mhango E.K.G.; Marín P.; Escudero E.; Yuste M.T.; Eiriksson F.F.; Snorradottir, B.S.; Sveinbjornsson B.R.; Gizurarson S. Bioequivalence study of lumefantrine rectal enema and the commercially available generic oral suspension. A pilot studies. *International Journal of Pharmaceutical Sciences and Research* **15** (5), In press, 2024 (Accepted manuscript, that will be published in May 2024)

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Declaration of Contribution

The data presented in this thesis was obtained in experiments conducted by the doctoral student, Ellen Kalesi Gondwe Mhango (EKGM), under the supervision of Sveinbjorn Gizurarson (SG).

Paper I: EKGM designed and conducted the study under the supervision of Professor SG. EKGM designed the design of experiment using MODDE®, performed the experiments and interpreted results under the supervision of SG and Bergthora S. Snorradottir (BSS). Benjamin R. Sveinbjornsson (BRS) did NMR work and interpreted the results. EKGM drafted the manuscript with help from coauthors and SG provided the overall direction.

Paper II: EKGM did research, calculations, contributed part of PK and PD changes, effect of malnutrition on PK of drugs, analyzed and discussed the work and drafted the manuscript. Dr Baxter H.K Kachingwe (BHKK) contributed Pk and PD changes during development. Dr Kondwani GH Katundu (KGHK) contributed effect of malnutrition in children, BSS proofread the manuscript. The general idea about the whole work was provided by SG who also did some calculations, discussed the work, and provided overall supervision of the whole work.

Paper III: EKGM wrote the experimental protocol under the supervision of SG. EKGM analyzed the data on the computer, interpreted and drafted the manuscript under supervision of SG and with help from other coauthors. Professor Pedro Marin (PM) applied for the ethical clearance in Spain and reviewed the experimental protocol together with Professor Elisa Escudero (EE) and EKGM. EKGM was trained on how to handle animals during blood sampling by PM and EE. Work on blood sampling was done by PM, EE, EKGM. Dr María Teresa Yuste (MTY) also provided support. Finnur Freyr Eiriksson analyzed plasma samples.

1 Introduction

The sad fact in our world today is that one child dies every minute because of malaria. In this section, the background for this project will be explored on some specific areas as well on areas that cover the work that was carried out in this PhD Thesis.

1.1 Malaria

Malaria is a parasitic disease spread by the bite of an infected female mosquitoes of the genus anopheles (Yun & Edginton, 2021). More than 120 plasmodium species exist, but only *plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale, Plasmodium malariae* and *Plasmodium knowlesi* are known for causing malaria in humans (Varo et al., 2020). The geographic distribution and various aspects of biology is different among these species (Abebaw et al., 2022). *P. falciparum* is the most infectious and is responsible for causing more than 99% malaria linked deaths (Varo et al., 2020). According to the World Health Organization (WHO) report 2021, during the Covid-19 pandemic, children's mortalities rose by 69,000 due to distractions in the supply of malaria prevention, diagnosis and therapy in Sub-Saharan Africa alone (World Health Organization, 2021). Universally, 405,000 mortalities were caused by malaria in 2018, and 94% happened in Sub-Saharan Africa. An overwhelming financial burden, US$ 3.1 billion, was due to these worldwide deaths (Madhav & Hoda, 2021; Varo et al., 2020). Universally, every minute, malaria kills a child. About 90% of deaths caused by malaria happen in Africa specially among children (Beavogui et al., 2020; Kabaghe et al., 2017). *Plasmodium falciparum* accounts for approximately 97% of all malaria cases (Dao et al., 2021). The rate of infection remains noteworthy in Sub-Saharan Africa in spite of strong funds and to reduce the incidence of malaria (Debash et al., 2022; Mwendera et al., 2019). The clinical manifestation of the disease can vary from without symptoms to severe or even deadly malaria. Malaria may lead to respiratory distress, impaired consciousness, low blood sugar, jaundice or even death (Abebaw et al., 2022; Adhikary et al., 2023; Chanda-Kapata et al., 2020). Symptoms include armpit temperature ≥ 37.5 °C (fever), headache, chills, vomiting, joint pains et cetera (Abebaw et al., 2022).

Malaria that does not present symptoms is likely to act as a reservoir of gametocytes. This makes parasites from carriers that do not present symptoms to be very infectious to the female mosquito and therefore, the main source of gametocytes for vectors of mosquitoes leading to persistence of malaria transmission (Abebaw et al., 2022). According to WHO guidelines, a patient is described as having uncomplicated *falciparum* malaria when he/she presents with symptoms of malaria and a positive

parasitological test but without features of severe malaria. A patient with severe malaria presents with one or more of the features explained in Table 1, happening in the lack of an alternate unknown cause in the presence of *P. falciparum* asexual parasitaemia.

Table 1. Symptoms of severe malaria reproduced from WHO guidelines for the treatment of malaria, 3rd edition. (https://www.afro.who.int/sites/default/files/2017-06/9789241549127_eng.pdf)

Symptoms	Explanation
Impaired consciousness	A Glasgow coma score <11 in adults or a Blantyre coma score < 3 in children
Prostration	Generalized weakness so that the patient is unable to sit, stand or walk without assistance
Multiple convulsions	More than two episodes within 24h
Hypoglycaemia	Blood or plasma glucose <2.2 mmol/L (<40 mg/dL)
Severe malarial anaemia	Haemoglobin concentration ≤5 g/dL or a haemacrit of ≤15% in children <12 years of age (<7g/dL and <20%, respectively, in adults) with a parasite count >10, 000/µL
Renal impairment	Plasma or serum creatinine >265 µmoL/L (3 mg/dL) or blood urea >20 mmoL/L
Jaundice	Plasma or serum bilirubin >50 µmoL/L (3 mg/dL) with a parasite count >100, 000/ µL
Acidosis	A base deficit of >8 mEq/L or, if not available, a plasma bicarbonate level of <15 mmol/L or venous plasma lactate ≥5 mmol/L. Severe acidosis manifests clinically as respiratory distress (rapid, deep, laboured breathing).
Pulmonary oedema	Radiologically confirmed or oxygen saturation <92% on room air with a respiratory rate >30/min, often with chest indrawing and crepitations on auscultation
Significant bleeding	Including recurrent or prolonged bleeding from the nose, gums or venepuncture sites; haematemesis or melaena
Hyperparasitaemia	*P. falciparum* parasitaemia >10 %
Shock	Compensated shock is defined as capillary refill ≥3 s or temperature gradient on leg (mid to proximal limb), but no hypotension. Decompensated shock is defined as systolic blood pressure <70 mm Hg in children or <80 mm Hg in adults, with evidence of impaired perfusion (cool peripheries or prolonged capillary refill)

1.2 Life cycle of the malaria parasite in human beings

When the infected mosquito bites a person, the parasite introduces sporozoites which go to the liver and multiply asexually within 7 to 15 days without causing clinical signs

and symptoms in what is known as the exoerythrocytic stage of development (Baer et al., 2007; Chanda-Kapata et al., 2020; Singh & Daneshvar, 2013; Yun & Edginton, 2021). Sporozoites mature to schizonts which rupture and release merozoites. Within red blood cells (RBCs), merozoites develop into trophozoites which upon attaining maturity multiply asexually forming schizonts that contain merozoites (Singh & Daneshvar, 2013). The schizonts in RBCs burst and release merozoites that continue to invade RBCs and this completes the erythrocytic cycle. This cycle gets repeated causing fever every time merozoites cause rupture of RBCs and invade more blood cells (Baer et al., 2007; Singh & Daneshvar, 2013; Yun & Edginton, 2021). Some merozoites do not replicate asexually but develop into male and female gametocytes which are sexual forms of the parasite. The gametocytes continue to develop when taken up by the female anopheles mosquito when it sucks blood (Baer et al., 2007; Singh & Daneshvar, 2013). The erythrocytic cycle duration varies according to the *Plasomodium* species, with *P. knowlesi* having the shortest cycle of about one day, *P. ovale*, *P. vivax* and *P. falciparum* having a cycle of about two days, while *P. malariae* has a duration of about three days. Therefore, if a patient is not treated, parasitemia can continue to rise approximately every day, every two days or every three days depending on the species involved (Singh & Daneshvar, 2013).

1.3 Approaches to control malaria

Globally, there has been efforts to reduce or eradicate malaria such as the Roll Back Malaria Initiative, which led to a great decrease of the burden in Sub-Saharan Africa from 2000 to 2015. This initiative was successful mainly because of indoor residual spraying (IRS) and the use of long-lasting insecticide nets (LLINs). In addition, early diagnosis, improved drug treatment and health infrastructure also helped (Adhikary et al., 2023; Mohammed-Awel et al., 2020).

Other initiatives include The Global Technical Strategy for malaria 2016 to 2030 which was approved in 2015 by the World Health Assembly aims to eradicate malaria by 2030 (Mohammed-Awel et al., 2020) and the *Zero X40* initiative which is an initiative of five chemical companies that are supported by the Bill & Melinda Gates Foundation and the Innovative vector Control Consortium. The Zero X40 initiative aims at eradicating malaria by 2040 (Willis & Hamon, 2018). These initiatives are also depending mainly on use of insecticides to control the parasite (Mohammed-Awel et al., 2020). Another initiative called the Medicines for Malaria Venture (MMV) aims to recommence and sustain the provision of cost effective antimalarials through the discovery, development and delivery of efficacious new medications for poverty stricken countries (Hentschel, 2002). *Despite these efforts, millions of children, especially in the tropical regions continue to be at risk (Adhikary et al., 2023)*. Malaria control is difficult because of the presence of carriers that do not present symptoms, the emerging of vectors that are resistant to insecticides, and the development of resistance to antimalarial drugs by the *Plasmodium* (Abebaw et al., 2022). In addition,

utilization of antimalarials of poor quality and the use of monotherapies have generally been associated with treatment failure in certain situations; thereby contributing to the development of resistance in some areas (Brock et al., 2018).

1.3.1 Prevention of malaria

Prevention of malaria is done in various ways but only the major and common methods have been discussed here.

1.3.1.1 Space spraying

Space spraying or outdoor spraying is a method that is used to spray a formulation containing an insecticide either by air or ground to kill the adult flying mosquitoes (Bonds, 2012). The formulation can be sprayed as an ultra-low volume (ULV) formulation (Bonds, 2012), fog (thermal fogging or cold fog) (Pryce et al., 2018) or barrier spray (Stoops et al., 2019) to kill adult mosquitoes. The minimum effective volume of the insecticide formulation that is sprayed without any further dilution is called ULV. However, if the formulation is diluted before spraying it is called low volume (LV) or high volume (HV) (Bonds, 2012). ULV is used to treat bigger areas due to its effectiveness and it kills mosquitoes faster because of the small droplet size. Organophosphate insecticides and various pyrethroid insecticides are used. In thermal fogging, the formulation is heated to make fog which is sprayed using a fogging machine to kill adult mosquitoes. This method has been used in emergency situations in response to epidemics of malaria, and in programs that control pests. Cold fog is also used. (Pryce et al., 2018) due to small droplet size produced, the method can be used to treat regions with thick vegetation. Formulations contain pyrethroid insecticides.

In back spraying, the formulation is sprayed on materials or the vegetation to control mosquitoes when they encounter the sprayed surface. Talstar which contains 7% bifenthrin is a product on the market currently available (Stoops et al., 2019). Space spraying is done using different types of machines such as truck mounted as well as using handheld sprayers and backpack sprayers. Aerial spraying which is the use of aeroplanes, helicopters and drones can also be used to kill mosquito larva and adult mosquitoes (Bonds, 2012; Chibi et al., 2023). However, space spraying only targets the adult flying mosquitoes which means that formulations need to be sprayed successively to kill adult mosquitoes that appear from the immature forms of the parasite such as larva stage. Another problem is that space spraying contaminates the ground for instance it has been reported that pyrethroids are a risk to some marine species (Bonds, 2012).

1.3.1.2 Indoor residual spraying (IRS)

Indoor residual spraying is a major pesticide vector control means for regulating and eradicating malaria. The use of IRS was initially established during the Global campaign

to eradicate Malaria from 1955 to 1969 by spraying dichlorodiphenyltrichloroethane (DDT) in addition to treating cases, management of the environment, and improvements in housing riks (Tangena et al., 2020). The Global Malaria Eradication Campaign (GMEP) had led to eradication of malaria in 37 countries by 1978. This achievement resulted to the extension of IRS utility in Africa and there has been achievements in various environments (Mathanga et al., 2012; Tangena et al., 2020). Carbamates, neonicotinoids, organochlorines, organophosphates and pyrethroids are the major classes of insecticides that WHO approved for IRS use. The little cost and lasting decay made DDT and pyrethroids very popular. Nevertheless, the development of resistance has led to the use of other pesticides, which can be more costly. (Tangena et al., 2020; Willis & Hamon, 2018).

1.3.1.3 Use of insecticide treated nets (ITNs) and long-lasting insecticide treated nets (LLINs)

Insecticide treated bed nets are mosquito nets that have pesticides designed to repel or kill mosquito parasites that encounter the net. There are two types of ITNs: nets that are treated conventionally by submerging them in the pesticides recommended by WHO. This type of net is supposed to be retreated after a certain period to ensure the sustained effect of the pesticides. The long-lasting insecticide nets (LLINs) which are treated in the factory by inclusion of the pesticides is another type of ITNs (Mangani et al., 2022). The net must maintain its effectual biological action for a minimum of 20 washes under standards established by WHO under settings of the laboratory and utilization under settings in the field. The use of ITNs or LLINs has been beneficial in limiting the burden of malaria by protecting people who sleep under them from parasite bites (Tizifa et al., 2018). However, the decline in efficacy of the ITNs and *Plasmodium* development of resistance to pyrethroid, a class of insecticides used in the nets are some of the limitations in fighting malaria and its extermination in the long term (Mathanga et al., 2012; Mohammed-Awel et al., 2020; Ngonghala, 2022). Pyrethroids are the choice of insecticides in nets because they are less toxic to human beings but very irritating to the adult malaria parasite (Mohammed-Awel et al., 2020). New types of ITNs have been recommended recently as they have been designed to have huge effect against the pyrethroids that are developing resistance to mosquitoes. The dual active ingredient (DAI) ITNs contain two insecticides, the pyrethroid combined with either a non-pyrethroid or an insect growth regulator (Barker et al., 2023). The DAI ITNs contain a pyrethroid - chlorfenapyr nets which contains a prethroid and a pyrrole insecticide to increase the effectiveness of the net in killing mosquitoes and the pyrethroid – pyriproxyfen nets which contains the pyrethroid and the IGR which interrupts the growth and reproduction of mosquitoes. Following this ITNs have recently been reclassified into three groups: available prequalified nets that contain pyrethroids only, pyrethroid + chlorfenapyr nets and pyrethroid + pyriproxyfen nets.

1.3.1.4 Intermittent preventive treatment of malaria in pregnant women

Malaria during pregnancy affects both the mother and neonate in countries where malaria is rampant. It affects over 11 million expectant women in Sub−Saharan Africa. Malaria in pregnancy leads to anemia, preterm births, miscarriage, low birthweight i.e., less than 2.5 kg as per WHO definition and even death (Figueroa-Romero et al., 2022; Mlugu et al., 2021). Intermittent preventive treatment of malaria in pregnant women (IPTp) using sulfadoxine pyrimethamine (SP) is used to prevent malaria during antenatal care visit starting at the second trimester in medium and extreme transmission areas (Figueroa-Romero et al., 2022; Mathanga et al., 2012). Nevertheless, the rise in resistance to SP in some areas where malaria is rampant is a problem. In addition, SP cannot be used in the first trimester of pregnancy and in women having HIV infection and are taking cotrimoxazole due to possible drug-drug interactions (Figueroa-Romero et al., 2022). Because of this, in the past years, other drugs were also studied so that they could be used instead of SP. However, among the studied drugs only dihydroartemisin piperaquine (DHA- PPQ) is the most promising alternative drug at present. However, there is a low uptake in some areas which is another challenge in malaria (Dun-Dery et al., 2021; Figueroa-Romero et al., 2022)

1.3.1.5 Intermittent Preventive treatment of malaria in infants

During Intermittent preventive treatment of malaria in infants, currently renamed as *perennial malaria chemoprevention* (PMC) full doses of an antimalarial drug e.g., stat doses of SP are given to infants at specific periods irrespective of whether they have malaria (Menendez et al., 2022). The WHO commended the use of IPTi in 2010 to prevent malaria in infants in countries where transmission is medium to extreme. Numerous trials in Sub−Saharan Africa showed that IPTi decreased the malaria burden by 30%. SP has been reported as an established approach to protect infants against malaria (Lahuerta et al., 2021). However, there has been inadequate adoption in these nations (Esu et al., 2019; Menendez et al., 2022). The inadequate adoption is due to the observation that the efficiency of the IPTi was uncertain as the efficiency results were denoted to the whole babyhood instead of only to the time when kids were being protected. Furthermore, the extensively branding of SP as an unsuccessful drug was constructed on the belief that molecular markers linked to decreased efficiency in managing malaria would forecast medication failure in infection prevention (Esu et al., 2019; Menendez et al., 2022). The policy on PMC that mirrors the extension of the Extended Program on Immunisation into year two of life was reviewed in June 2022 by the WHO (Menendez et al., 2022).

1.3.1.6 Use of insect repellant products

Although repellants don't offer complete protection, they can decrease the chance of getting the disease. Different insect repellant products are used (Bibbs & Kaufman, 2017; Peng et al., 2022; Rodriguez et al., 2015).

1.3.1.7 Other preventive measures

Draining stagnant water around homes especially after heavy rains and emptying containers, maintenance and cleaning of drains, putting screens on windows and doors to prevent mosquitoes from entering homes, wearing long sleeved shirts and pants, shunning outdoor activities during hours when mosquitoes are at peak (Castro et al., 2010; Keiser et al., 2005; Tizifa et al., 2022) are some of the measures that are undertaken.

1.3.1.8 Malaria vaccine

The RTS, S/AS01 (Mosquirix™) is the first malaria vaccine that has been approved to fight malaria. GlaxoSmithKline (GSK), a British pharmaceutical company, developed the vaccine, and a pilot program was implemented in Ghana, Kenya and Malawi (El-Moamly & El-Sweify, 2023). The WHO in October 2021, commended the use of the Mosquirix RTS,S/AS01 vaccine for broader utilization together with existing tools used to prevent and control the spread of malaria in children living in areas of medium to extreme high transmission. (Menendez et al., 2022). The children vaccine acts against *P. falciparum* by decreasing the frequencies children contract malaria plus severe sickness and decreasing child mortality. The vaccine is a recombinant vaccine, consisting of the circumsporozoite protein (CSP) on the *Plasmodium falciparum* from the pre-erythrocytic stage. When this vaccine is introduced to children it induces antibodies CSP on the parasites, so when the parasite enters the circulation the antibodies will recognize the CSP and label the surface for the macrophages to induce an attack (Laurens, 2020). These antibodies can prevent the invasion of parasites into the hepatocytes, and it also elicits a cellular response enabling the destruction of infected hepatocytes. The capability of the parasites to mature in the liver and cause clinical malaria is therefore decreased. However, the prevention offered by the vaccine is not perfect as it is approximately 50% effective protective in 5 to 17 months year old children with clinical malaria in the initial year after vaccination. After a period of four years efficiency falls to 36% for episodes that are clinical and 32% for severe disease. During testing in infants aged from 6 to 12 weeks the efficiency of the vaccine was inferior and benefits were considered insignificant to explain its usage in this group. In comparison to the polio or measles vaccine which are greater than 90%, the efficiency of the current malaria vaccine is unsatisfactory (Moorthy & Okwo-Bele, 2015).

The WHO strategic advisory group of experts (SAGE) and the malaria policy advisory group (MPAG) approved a second vaccine, the R21/Matrix-M malaria vaccine for preventing malaria in children in 2023. Its mode of action is same as the RTS, S/AS01 vaccine by stimulating an immune response to circumsporozoite protein antigen present on surface of the *P. falciparum* sporozoite that are injected by mosquitoes. This vaccine utilizes the Novavax's adjuvant technology to boost the body's immune response. The efficacy of the vaccine was 74 % and upon doubling the dose it was 77% and the great efficacy was continued to 12 months in children who were given the

highest dose. A follow up trial showed that efficacy continued for a period greater than two years (Stertman et al., 2023). The vaccine was developed by Jenner Institute at Oxford University and Serum Institute of India. Currently it has been licensed for use in Burkina Faso, Ghana and Nigeria. It has been reported that, when used in combination with other measures of public health like use of ITNs, the vaccine can assist in improving and saving the lives of children.

1.3.1.9 Chemoprophylaxis of malaria

Atovaquone/proguanil (malarone), mefloquine, and doxycycline are examples of drugs that are recommended to be taken by those going to regions where malaria is very endemic (Genton & D'Acremont, 2012). A usual method that is employed is known as the "A, B, C, D" rule for prevention of malaria; whereby the four letters stand for **A**wareness of risk, **B**ite avoidance, **C**ompliance with chemoprophylaxis, and prompt **D**iagnosis in the event of fever. The risk of getting malaria depends on the exposure time and the strength of malaria transmission (Genton & D'Acremont, 2012).

1.3.1.10 Emerging techniques to control malaria

New control measures are emerging to control mosquitoes which include genetic control, incompatible insect technique (IIT), sterile insect technique (SIT), use of adult mosquito control traps, lethal ovitrap and autocidal gravid ovitrap (AGO), larvicide traps, auto dissemination method, endectocides, and attractive toxic sugar bait (ATSB).

Genetic control

Genetic abnormalities are introduced into the eggs of wild mosquitoes to either eradicate mosquitoes or to make them less capable of transmitting main pathogens as a result of some recent progress in genome based editing of the CRISPR/Cas9 (Bernardini et al., 2018; Caragata et al., 2020; Wang et al., 2021). Interest genes of mosquitoes can also be suppressed by taking advantage of the RNA interference (RNAi). Various genetically altered strains of *Aedes aegypti* (OX513A) have been developed by Oxitech company. Some strains of mosquitoes such as the OX513A have been altered genetically to make the female offspring die in the larva period, and therefore avoiding emergence of adult mosquitoes (Airs & Bartholomay, 2018; Evans et al., 2019). In 2021, another strain, the OX5034 was tested (Spinner et al., 2022). However, there has been concerns that mutations could occur in genetically modified (GM) mosquitoes that upon reproduction can cause a new threat to humans. In addition, it is very expensive and takes a long period of time to produce GM mosquitoes.

Sterile insect technique (SIT)

Sterile male mosquitoes are released in huge numbers into the wild and enter competition with the wild male mosquitoes to mate with female mosquitoes. Mating with

sterile male prevents the female mosquito from producing offsprings and this cause a reduction in the population of the next generation (Alphey et al., 2010). One of the many concerns is that the method is too slow and can only be done over large areas.

Adult mosquito control traps

Various designs of mosquito traps have been made to either directly kill mosquitoes using electric wires or killing collected adult mosquitoes. Adult mosquitoes are attracted to the traps by ultraviolet (UV), light emitting diode (LED), regular light and other attractants and thereby die due to electric shock, pesticides, sticky pads and other mechanical means. The type of trap to be used depends on the region, species being targeted, economy and availability of power supply (Gorsich et al., 2019; Kline, 2006).

Lethal ovitrap and autocidal gravid ovitrap (AGO)

The AGO trap by CDC has been utilized to decrease the population of mosquitoes by targeting the female mosquitoes when they are looking for an area where they can lay eggs (Barrera et al., 2014).

Larvicide traps

After eggs have been hatched, the larva is usually killed by using ovitraps which controls mosquitoes that only inhabit the containers. A modification of these traps has been made to restrict the larvae in the containers after eggs have been hatched and this kills the new adult mosquitoes that emerge (Snetselaar et al., 2014; Talbalaghi et al., 2020).

Auto dissemination method

Employ the use of insect growth regulator (IGR) such as pyriproxyfen through egg laying female mosquitoes for spreading to containers in which they breed and water bodies to kill mosquito at larval phase (Seixas et al., 2019). Auto dissemination by male mosquitoes is also possible through us of IGRs and other insecticides (Brelsfoard et al., 2019; Mains et al., 2015). Therefore, both female and male mosquitoes act as carriers of insecticides to be dispersed (Unlu et al., 2017). Methoprene is also an IGR that has been reported to control larvae in the laboratory (Bibbs et al., 2016). In2Care is another type of traps that has been reported to be used in auto dissemination (Buckner et al., 2017).

Endectocides

Involve administration of drugs to humans and animals so that when the mosquito and other insects suck blood they take in the drug that kill them. Available oral products are only administered to dogs and cats to kill mosquitoes and fleas (Foy et al., 2011).

Attractive toxic sugar bait (ATSB)

Mosquitoes are made to feed on a sugar formulation that contains a toxic ingredient. Spraying of ATSBs on vegetation can control mosquitoes (Chiu et al., 2024; Njoroge et al., 2023; Traore et al., 2020).

1.3.2 Treatment of P. falciparum malaria

The major objective of therapy is to achieve the swift and complete elimination of the *plasmodium* parasites from a patient's circulation to avoid an uncomplicated malaria from developing to severe infection or death. If malaria is managed effectively, transmission of the disease is reduced. Antimalarial drugs are used to prevent the infection from developing or to treat the disease. Apart from pregnant women, ACTs, are the drug of choice for treatment of uncomplicated malaria. Numerous drugs and drug combinations are recommended by WHO to be used to treat malaria, but only drugs that have been used in this project will be described in detail.

1.3.2.1 Artemisinin and its derivatives

Artemisinin (qinghaosu) was derived from *Artemisia annua*, a Chinese herb and belong to a class of sesquiterpene trioxane lactone. It acts by forming free radicals through separation of the endoperoxide bond in structure (Ho et al., 2014). The resulting free radicals slow down the synthesis of proteins during development of trophozoites (Nasir et al., 2012). Artemisinin has also been reported to work through inhibition of oxygen utilization by the parasites (Krungkrai et al., 1999). Artemisinin for commercial use is still obtained from the plant even though numerous efforts have been made to get the drug through full chemical synthesis utilizing bioengineered microorganisms (Li & Wu, 2003; Zeng et al., 2008). Artemisinin from plant harvest differ greatly according to growth environments (Chaturvedi et al., 2010). Drugs derived from *artemisia annua* are regarded as the keystone in the treatment of malaria caused by *P. falciparum* because of their potency and quick action. Regimens centered on artemisinin have gametocytocidal properties and therefore, inhibit the transmission of parasites (Murambiwa et al., 2011). Artemisinins are active against almost all asexual and sexual phases of the malaria parasites (Cui & Su, 2009).

Even though, artemisinin showed potential in treatment of malaria, it has certain drawbacks for instance solubility is low, poor bioavailability and short half-life (Zeng et al., 2023). Therefore, *semi synthetic derivatives* such as artemether, artesunate and dihydroartemisinin (DHA) were synthesized (O'Neill, 2005). However, the limitation of semi synthetic derivatives is that they need artemisinin as the commencing material, and even though full synthesis of artemisinin has been accomplished, the process is extremely extensive for cost effective production. To overcome these challenges, various researchers have produced antimalarials that are fully synthetic (O'Neill, 2005). Artemether is a methyl ether derived from artemisinin. It kills blood schizonticides and

gametocytes (Murambiwa et al., 2011). Artemether works by producing free radicals that delay the synthesis of proteins during development of the parasites (Byakika-Kibwika et al., 2011; Cui & Su, 2009). Artemether is administered either as a fixed dose combination with lumefantrine orally or as monotherapy, intramuscularly, dissolved in oil (Nyunt & Plowe, 2009). Following oral administration, AR undergoes rapid absorption with peak plasma concentration attained in about 2h and is quickly metabolized to its main active metabolite DHA (Patel et al., 2024).

Artemether is reported to be less active than DHA (Silamut et al., 2003). AR has a short half-life of about 2 to 3h (Waller & Sampson, 2018). There are different reports concerning the enzymes responsible for the metabolism of AR such as CYP3A4, CYP2B6 and CYP2C11 (Nyunt & Plowe, 2009); CYP1A2, CYP2B6 and CYP3A4 (Ali et al., 2010). Other reports indicate that AR is predominantly metabolized by CYP3A4/5 in the liver and elimination of both AR and DHA occurs in plasma. AR is highly protein bound about 95% *in vitro* (Patel et al., 2024). Following IM administration, AR undergoes slow, poor and greatly erratic absorption (Esu et al., 2014; Karbwang et al., 1997; Mithwani et al., 2004; Murphy et al., 1997). The IM dosage form is available for both adults and children. The initial IM dose is 3.2 mg/kg whereas the maintenance dose is 1.6 mg/kg IM daily until the patient can take oral medication (Esu et al., 2014). The physicochemical properties of artemether are listed in Table 2.

Table 2. Physical chemical properties of artemether and lumefantrine

Physicochemical property	Artemether	Lumefantrine	Reference
Molecular weight (g/mol)	298	528.94	(W. Lin et al., 2016; Olafuyi et al., 2017)
Log *P*	3.28	8.7	(W. Lin et al., 2016; Olafuyi et al., 2017)
pKa 1	None	14.1	(Wen Lin et al., 2016; Olafuyi et al., 2017)
pKa 2	None	9.80	(W. Lin et al., 2016; Olafuyi et al., 2017)
B/P	0.8	0.8	(Wen Lin et al., 2016; Olafuyi et al., 2017)
Free fraction (unbound) in plasma	0.05	0.003	(Colussi et al., 1999)
Solubility in water (mg/ mL)	0.012	0.002	(Kotila et al., 2013)

1.3.2.2 Amino alcohols

Lumefantrine (LF), halofantrine and mefloquine are related compounds where LF (previously called benflumetol) was discovered in China (Deen et al., 2008). LF works by detoxifying hemoglobin in the parasite and kills blood schizonticides (Byakika-Kibwika et al., 2011; Cui & Su, 2009; Murambiwa et al., 2011). The absorption of LF is slow and the bioavailability is very variable and depends on intake of a fatty meal. An equivalent of 1.2 g fat or 36 mL soya milk is required to achieve 90% absorption in healthy subjects (White, 2010, 2014). In the acute phase of malaria, LF absorption is decreased, but rises considerably when symptoms start to resolve, and the patient starts to feed. Variation in the fat content of the meal is much more likely to affect the bioavailability of orally administered LF than AR; e.g. the bioavailability of LF when taken with maize porridge plus vegetable oil (high fat diet) was similar to when taken with milk but higher than when taken with maize porridge alone (low fat diet) (Brody, 2018; Mwebaza et al., 2013). LF undergoes liver metabolism to its major metabolite, desbutyllumefantrine (DBL) and the main routes of excretion is in bile and feces (White, 2014). The antimalarial activity of DBL is greater but its contribution to overall antimalarial effect is relative (White, 2014). In comparison with other partner drugs used in ACTs, LF has never been used as monotherapy in the treatment of malaria. The physicochemical properties of LF are listed in Table 2. Limited data on the PK of AR and LF in children exist as shown in Table 3.

Table 3. The pharmacokinetic parameters of artemether and lumefantrine, based on values obtained from WHO (guidellines for the treatment of malaria, 3rd edition and literature (https://www.afro.who.int/sites/default/files/2017-06/9789241549127_eng.pdf).

	Artemether			**Lumefantrine**			
Parameter	**Adults**	**Children**	**Malnourished Children**	**Adults**	**Children**	**Malnourished Children**	**Comments**
Dose (mg/kg)	1.14	5		6.86	29		
C_{max} (ng/mL)	100	119		7.91 **		15% lower	Food increases C_{max} for AR 2-3-fold
t_{max} (h)	2.0			6			
$t_1/2$ (h)	1.9	16		95	123	12% shorter	Malaria makes half-life significantly longer (LF)
AUC (ng·h/mL)	320	392		207		31% lower	Malaria increases AUC significantly (AR)
V_d (L/kg)	6.05	0.9		3.8/0.71	1.3		
Cl (L/h per kg)	0.91						3-fold lower Cl in <3 months vs >3 months (AR)

	Artemether			Lumefantrine			
Parameter	**Adults**	**Children**	**Malnourished Children**	**Adults**	**Children**	**Malnourished Children**	**Comments**
F (%)	43			65			Lower in fasted subjects, or age <5 year (LF)
fu (%)	4.6			0.3			
Drug Interactions	↑			↑			
Effect of ketoconazole	↑			↑			
Effect of mefloquine	NE			↓			
Effect of quinine	↓			NE			
Effect of lopinavir/ritonavir	↓			↑			

** Food may increase the absorption of lumefantrine up to 16—fold due to increase in bile salts. Units for LF are (µg/mL). NE = No effect, AUC = Area Under the Curve, V_d = volume of distribution, t_{max} = Time to maximum plasma concentration, Cl = Clearance, ↑ = increased plasma concentration or exposure, ↓ = decreased plasma concentration or exposure.

1.3.2.3 Artemisinin combination therapy (ACT)

The WHO recommended use of ACTs as the first line medication for uncomplicated malaria in 2006 and have continued as the pillars in the treatment of malaria (Bassat et al., 2015; Lyu et al., 2021; Mathanga et al., 2012). ACT is a blend of a quick-acting artemisinin derivative and a partner drug. This blend is established on the piece of evidence that the rapid, brief acting artemisinin derivative reduces the number of malaria parasites rapidly while the slower, extended acting partner drug eliminates the residual parasites from the blood and safeguards artemisinin derivatives from developing resistance (Prabhu, Suryavanshi, Pathak, Sharma, et al., 2016). The ACTs in Table 4 have been commended for use in children and adults excluding expectant women in the initial trimester. However, focus will be on AR and LF as they were chosen as model drugs on this project.

Table 4. ACTs recommended by WHO (https://www.afro.who.int/sites/default/files/2017-06/9789241549127_eng.pdf)

Artemisinin derivative	Companion drug
Artemether	Lumefantrine
Artesunate	Amodiaquine
Artesunate	Mefloquine
Dihydroartemisinin	Piperaquine
Artesunate	Sulphadoxine – pyrimethamine

AR–LF is one of the ACTs that was recommended by WHO to treat uncomplicated *falciparum* malaria. According to the 3rd edition of the WHO guidelines for the treatment of malaria, the dose range for artemether is 5–24 mg/kg and for lumefantrine it is 29–144 mg/kg. The recommended dosage regimen shown in Table 5 is given every 12 hours for 3 days, with the first two doses administered 8 h apart. The total number of doses are six.

Table 5. Recommended dosage regimen for artemether and lumefantrine, reproduced from WHO guidelines for the treatment of malaria 3rd edition (https://www.afro.who.int/sites/default/files/2017-06/9789241549127_eng.pdf).

Body weight (kg)	Dose (mg), artemether + lumefantrine
5 to < 15	20 + 120
15 to < 25	40 + 240
25 to < 35	60 + 360
≥ 35	80 + 480

WHO recommends use of intravenous (IV) artesunate for at least 24h in treating severe *falciparum* malaria in children until they can tolerate oral ACTs. In the absence of IV artesunate, use of intramuscular (IM) artemether is preferred to quinine. When absolute treatment of severe malaria is impossible in children suspected of having severe malaria and waiting to be referred to a higher-level facility, they are given a single dose of IV artesunate or IM artemether when IV artesunate is unavailable. If IM artemether is also not available, IM quinine is given. When IM artesunate is unavailable, children below 6 years of age are given a single dose of rectal artesunate and immediately referred to the next level of health care. However, rectal artesunate is not given to older children and adults. Some researchers tried to formulate AR–LF nanostructured lipid carriers (NLC) for intravenous delivery (Prabhu, Suryavanshi, Pathak, Patra, et al., 2016), NLC for oral therapy (Prabhu, Suryavanshi, Pathak, Sharma, et al., 2016) and AR–LF lipid for parenteral use (Ma et al., 2014). There is lack of parenteral dosage forms of ACTs commercially and therefore, the available ACTs recommended by WHO are given orally.

1.4 Resistance to antimalarial therapy

The ability of a parasite to survive or multiply in spite of a therapeutically administered dose of medication is called resistance (Cowell & Winzeler, 2019). Prevention and treatment of malaria is challenged by the development of resistance. *P. falciparum* is the malaria parasite with pronounced possibility of drug resistance (Conrad & Rosenthal, 2019). Resistance to antimalarial drugs has been reported in three of the five species that infect humans namely *P. vivax*, *P. malariae* and *P. falciparum (White, 2004)*. Resistance has been reported to all antimalarial drugs including derivatives of artemisinin (Achan et al., 2011). Improper utilization of antimalarials causes a strong selective pressure on malarial parasites to develop great levels of resistance. In addition, utilization of antimalarials of poor quality and the use of monotherapies have generally been associated with treatment failure in certain situations; thereby contributing to the development of resistance in some areas (Brock et al., 2018). Increased levels of resistance lead to lasting parasitaemia and ultimately treatment failure and death (Cowell & Winzeler, 2019; White, 2004). Utilization of combination drugs with different modes of action and adherence to right dose regimens can prevent or delay the development of resistance (Cowell & Winzeler, 2019). The WHO recommended use of ACTs to prevent or delay the development of resistance to antimalarial drugs.

1.5 ACT pediatric dosage forms

Treating certain diseases in children is a very big challenge because of the absence of suitable dosage forms for this age group. Children aged younger than six years of age struggle to swallow oral dosage forms such as tablets and capsules (Del Moral Sanchez et al., 2018; Trastullo et al., 2015; van Riet-Nales et al., 2015). The problem is even

worse if these children have severe malaria and/or are unconscious as they cannot swallow any oral medication. Children aged younger than three years of age were reported to have passed on as a result of chocking, following swallowing tablets of albendazole in the course of mass drug administration campaigns (Trastullo et al., 2015). That being the case, tablets are at times either cut or crushed to prepare a suspension that is administered in conjunction with a drink or food to enhance compliance. This may result in measurements that are inaccurate or confer a stability issue (Liu et al., 2015; Salunke & Tuleu, 2018). Many of the ACTs that have been recommended by WHO are available commercially as oral dosage forms such as tablets and suspensions. However, Children with severe or cerebral malaria are unconscious, have seizures or may be vomiting and therefore oral dosage forms may not be an option. As a result, these children die due to malaria. The rectal ACTs in form of suppositories would not be applicable in some countries which are very hot in the sub-Saharan region. In addition, the limited volume of fluid available for dissolution in the rectum makes suppositories not a very ideal means of delivering drugs especially the very poorly water-soluble drugs. Therefore, there is a need for an alternative dosage form such as a rectal enema designed for children under five years of age that can be used and stored at room temperature even in rural areas in the tropical climate. In this scenario, rectal enema may be an alternative as the drugs will remain in solution and therefore disintegration and dissolution stages that solid dosage forms undergo can be bypassed.

1.6 Anatomy and physiology of the rectal route of humans

The rectum, being the last part of the large intestine, is mainly responsible for transporting or as a short-term storage facility during the defecation process. The rectum keeps feces if it is little in volume till the rectum become distended enough for defecation to start (Hua, 2019). Even though the rectum is created at birth it starts to function when the baby starts to feed by mouth (Jannin et al., 2014). The rectum is an extension of the sigmoid colon and continues to the anal canal at the anorectal intersection. It is housed within the pelvis high up the level of the pelvic floor (Bazira, 2023).

The inside of the rectum is lined by a layer of mucus (rectal mucosa) which is approximately 150 μm thick and is in contact with faeces (Nunes et al., 2014; van Hoogdalem et al., 1991). This mucus layer is primarily water and mucin (Hua, 2019; Johansson et al., 2013; Pullan et al., 1994). The thick muscle of the rectal wall projects inwards and overlays the mucosa (Bazira, 2023). The surface of the rectal mucosa is made up of a single layer of columnar cells that form the epithelium (simple columnar epithelium) (Hua, 2019; Tanaka et al., 2012) as seen in Figure 2. The goblet cells of the epithelium secrete mucus that protect the epithelia of the rectum and also assist in lubricating feces as it gets transported out of the rectum (Hua, 2019). The turnover time for this fluid layer is approximately 3–4 h (Hua, 2019; MacDermott et al., 1974). The

rectum lacks villi and microvilli and therefore has relatively limited surface area when compared with the small intestine (Nunes et al., 2014; van Hoogdalem et al., 1991).

Various researchers have debated the exact length of the rectum. The rectum of adult humans is reported to be 12 cm long (Chiva & Magrina, 2018), 10 to 14 cm long (Bazira, 2023), 12 to 15 cm long (Shroyer & Kocoshis, 2011), 12 to 18 cm long (Barleben & Mills, 2010), and Salerno et al. reported a length of 15 cm long (Salerno et al., 2006). Other researchers reported that, in adults the rectum is approximately 15 to 20 cm long and a surface area of about 200 to 400 cm^2 (Bharucha & Klingele, 2005; de Boer et al., 1982; Kenig & Richter, 2013; Nunes et al., 2014; Sýkora et al., 2012). The rectum can be split into three segments from the anal verge namely lower (0–6 cm), middle (7–11 cm) and upper (12–15 cm) rectum (Salerno et al., 2006).

However, in infants and children the length depends on age. The volume, length and diameter of the rectum in infants continue to develop till it reaches the size of an adult generally at 10 years of age (Anderson & Saneto, 2012). For instance, at the age of one month the rectum is approximately 3 cm long with a surface area of about 18 cm^2 while at the age of 10 years the rectum is approximately 12 cm long and surface area of about 230 cm^2 as shown in Table 6. The rectal data were collected from a study done in normal infants and children at Arkansas Children's hospital in the United States of America (Jannin et al., 2014; Woody et al., 1989).

Table 6. Rectal size changes with age (adapted from Woody et al. and Jannin et al.)

Age	*Diameter (cm)*	*Length (cm)*	*Surface area (cm^2)*	*Volume (mL)*
1 mo	1.5	3	18	7
3 mo	3.0	6	71	42
1 yr	3.5	7	96	67
2 yr	4.0	8	126	100
6 yr	4.5	9	159	143
10 yr	5.0	12	228	235

Venous drainage of the rectum occurs via three veins. The upper part of the rectum is drained by the superior haemorrhoidal veins which empties into the portal venous system. The venous drainage of the lower part of the rectum is via the inferior and middle haemorrhoidal (rectal) veins which empty into the systemic circulation, and this partly avoids the first pass effect by the liver. In addition, the lymphatic drainage which also bypasses hepatic first pass metabolism, drains the rectum to a larger extent which

could raise the absorption of drugs that are very lipophilic (Jannin et al., 2014). Figure 1 shows comparative anatomy of the rectum of a rabbit and human.

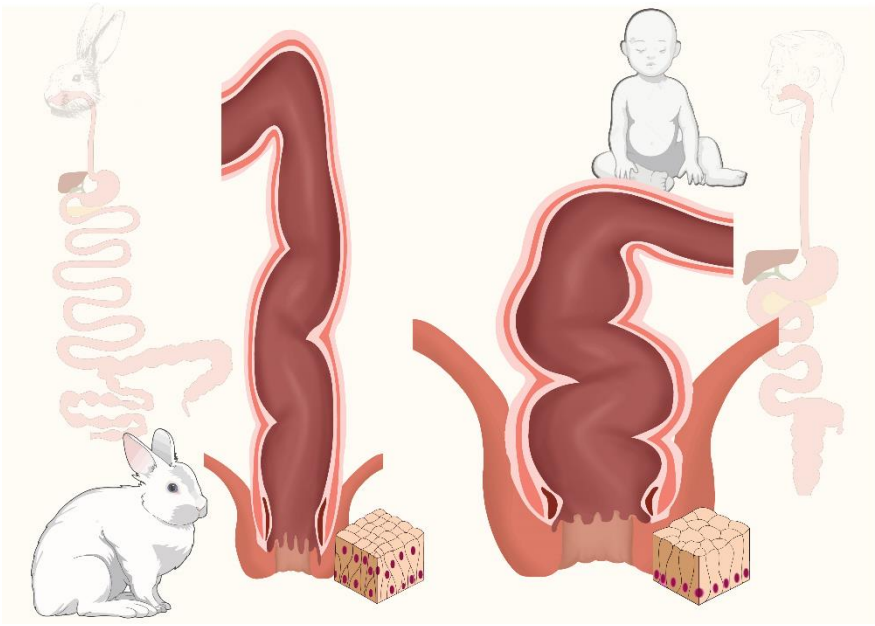

Figure 1. Comparative anatomy of rabbit and human showing the rectal mucosa. From original publication by EKG Mhango et al. 2024

The rectal environment is relatively stable compared to other regions of the gastrointestinal tract (GIT) making absorption to be reproducible with a mean fluid volume of about 1-3 mL. The limited volume of fluid may be a challenge to the dissolution of poorly soluble drugs. The buffering capacity of the fluid in the rectum is little or negligible with pH of 7–8 (Jannin et al., 2014; Nunes et al., 2014; T. J. Purohit et al., 2018). Differing reports regarding the pH of the rectum in children exist. The average pH in the rectum of healthy infants and children was reported to be 9.6 with a big range of 7.2–12.1 (Jantzen et al., 1989). The mean pH of 6.75 from healthy and sick infants was reported without any significance difference between the groups (Turner et al., 2012). On the other hand, the average rectal pH of healthy neonates is reported to be 6.47 compared with pH, 6.9 in infants who are older than 28 days (Turner et al., 2012). Enzymatic activity is reported to be low within the rectum as compared to other regions of the GIT (Hua, 2019). Increased motility of the bowel such as in diarrhea can decrease the time the formulation is retained in the rectum which can lead to less drug being released and absorbed (Rathi et al., 2022). The same thing can happen if the formulation is irritating.

1.7 Rectal drug delivery systems

Even though the oral route of medication administration is the most convenient, there are situations where this is impossible. In these situations, rectal drug delivery may be an alternative for administration of drugs to attain either local or systemic effects (Hua, 2019). Products that have no therapeutic properties are also given rectally to achieve various purposes such as lubrication, to clean the colorectum prior to some medical procedures such as examination of the colon (Melo et al., 2018). Rectal drug delivery systems can be grouped into conventional and novel dosage forms. Conventional rectal formulations can be classified into three groups: solid dosage forms such as suppositories, capsules etc; gels, ointments, creams are examples of semisolid dosage forms; liquid dosage forms such as solutions, emulsions, suspensions (Trusha J. Purohit et al., 2018; Rathi et al., 2022). The volume of administration may be from 2.5 mL to a few hundred mL. If the volume is between 5 to 10 mL the liquid formulations are known as microenemas (T. J. Purohit et al., 2018). Enemas are usually utilized to stimulate the rectum to start off defecation prior to procedures requiring operation (Pittet et al., 2015), and to offer local treatment in conditions such as ulcerative colitis or inflammatory bowel disease (Matuszyk et al., 2016), and for diagnostic purposes (T. J. Purohit et al., 2018). Enemas have also been used for acute treatment such as epileptic seizures (Seigler, 1990).

The difference between novel rectal and conventional dosage forms is in the properties of the formulations. Novel formulations provide improvement in drug solubility, extended retention, sustained drug release and spreadability of the formulation can be controlled (T. J. Purohit et al., 2018; Rathi et al., 2022). Examples of novel rectal dosage forms include: thermoresponsive also known as thermoresponsive liquid suppositories or simply liquid suppositories whose base (polymer) turns into a gel at physiological temperature (37 °C) and release the drug slowly (Lin et al., 2012); hollow type (HT) suppositories have a hollow cavity in which solid, gel or liquid can be housed (Matsumoto et al., 1993; Shiohira et al., 2009; Watanabe et al., 1998). The drug can be loaded in both the shell and cavity which offers quick release of the drug from the cavity followed by slow release from the shell.

The other advantage of HT suppositories is that the drug can be incorporated in the shell as well as in the hollow cavity, which can provide rapid drug release from the core followed by sustained release from the shell (T. J. Purohit et al., 2018; Shiohira et al., 2009); mucoadhesive gels have been used to increase the residence time of drugs in the rectum thereby extending drug release (Gaudin et al., 2008; Ryu et al., 1999) and also leading to a decreased formation of toxic metabolites (Xu et al., 2017); micro and nanoparticles have also been utilized to achieve targeted and sustained drug release by loading them into either a suppository base or a gelling polymer as vehicles (Basavaraj et al., 2013; Maisel et al., 2015). Other novel rectal dosage forms include microspheres, liposomes (Rathi et al., 2022), niosomes (Kamel et al., 2013), solid lipid

nanoparticles (SLN) (Abdelbary & Fahmy, 2009), nanomicelles (Seo et al., 2013) etc. Using extended release is limited by the defecation process.

Rectal drug delivery may be very helpful in the following situations: nauseous and vomiting patient, unconscious patient, patient with swallowing difficulties, drugs that cause GIT irritation, drugs that degrade at gastric pH, drugs that undergo extensive metabolism in the liver, noninvasive and can be administered by unskilled healthcare personnel (Rathi et al., 2022). Rectal drug delivery is sometimes forsaken due to the following limitations: limited fluid for dissolving drugs that have poor water solubility, small surface area for absorption, variable absorption, and poor acceptability in some regions of the world. When designing dosage forms for rectal delivery, several factors need to be considered which can be from both the pharmaceutical and physiological perspective (Hua, 2019). The rectal mucosa has limited transport capacity and the rectal formulations must be inserted in the lower segment of the rectum to prevent absorption via the superior rectal vein (Jannin et al., 2014).

The buffering capacity of the fluid in the rectum is little so, formulations administered can change the pH in the rectal region significantly (Jannin et al., 2014; Nunes et al., 2014; T. J. Purohit et al., 2018). Changes in pH can affect drug absorption, cause irritation or harm the mucosa (Nunes et al., 2014). Therefore, this needs to be considered when designing rectal formulations for drug delivery to be effective. Drug partitioning and absorption can be affected by the pH of the rectum (Hua, 2019). Drugs with pKa values close to or higher than the reported range are preferentially absorbed because rectal pH is considered neutral (Jannin et al., 2014; T. J. Purohit et al., 2018). It is reported that drug degradation and metabolism presystemically, including enzymes secreted by local bacteria in the rectum is not significant (De Boer et al., 1984; Jannin et al., 2014; Macfarlane & Macfarlane, 2011; Sartor, 2008). Relatively limited absorption takes place in the rectum due to absence of villi and microvilli (Nunes et al., 2014; van Hoogdalem et al., 1991). However, numerous advantages of rectal drug delivery exist.

Retention of drug in the rectum for a sufficient duration is required for drugs to be absorbed both locally or systemically. On the other hand, leakage of the formulation can be a problem (Hua, 2019; Nunes et al., 2014). Inclusion of mucoadhesives or adjustment of the viscosity may modify spreading of the product which also avoids the drug to be drained by the upper rectal veins (Jannin et al., 2014). Following administration, drugs need to be released from the formulation and get or stay dissolved in the rectal fluid (Nunes et al., 2014). After dissolution, drugs need to go through a barrier, the mucus layer which is approximately 150 μm thick to access the epithelial cells that line the rectum to be absorbed (i.e., to get into blood and/or lymph). Therefore, the duration the drug remains in contact with mucosa of the rectum is critical for the absorption process. The mucus layer, which is a fluid layer, is primarily

water and mucin (Hua, 2019; Johansson et al., 2013; Pullan et al., 1994). The turnover time for this fluid layer is approximately 3–4 h (Hua, 2019; MacDermott et al., 1974).

The viscosity of the contents in the rectum can be altered if feces are present in the rectum. This also can modify the dissolving process, stability of the drug and contact of the drug with mucosa during absorption (de Boer et al., 1982; Hua, 2019; van Hoogdalem et al., 1991). This can cause drugs to interact with the stool and mucus in a non-specific manner resulting in absorption of drugs to be irregular. In addition, due to defecation, drugs can be expelled resulting in incomplete absorption. Therefore, dosing time with regard to the bowel movements of the patient must be considered (Sathyan et al., 2000) if possible. A decrease in retention of the formulation can cause drug release to be incomplete as a result of an elevated motility of the colon when children have diarrhea (de Boer et al., 1982; Hua, 2019; van Hoogdalem et al., 1991). Several pathological conditions including inflammation of the mucosa, trauma etc. can modify drug absorption (Hua, 2019). However, rectal drug delivery possesses several advantages compared to oral drug delivery.

During preformulation It is important to consider the solubility of the drug. When formulating liquid dosage forms, the bioavailability of drugs with limited solubility can be improved by using self-emulsifying drug delivery systems (SEDDS). SEEDS consist of an oily base and a surfactant. A hydrophilic cosolvent or cosurfactant may or may not be added. Upon contact with water at the site of administration, SEEDS transform to an oil in water emulsion. It is also reported that SEDDS improve the permeability of membranes (Cherniakov et al., 2015).

Drug absorption is faster with liquid formulations because drug release and dissolution stages are bypassed (Hua, 2019). However, the composition of the enema can have an influence on rectal drug delivery. Higher drug retention in the rectum is achieved with use of small volumes whereas volumes greater than 80 mL can provoke defecation; or low volume where the compound or the formulation induces irritation (Nunes et al., 2014; van Hoogdalem et al., 1991). The extent of leakage from different types of liquid formulations is different and can cause drug absorption to be irregular (Hua, 2019).

1.8 Rectal drug delivery of ACTs

Rectal administration of artemisinin and its derivatives has been tested in various studies to treat severe malaria in rural areas. Available drugs that have been used in rectal formulations include artemisinin, artesunate, artemether and dihydroartemisinin (Gomes et al., 2008). Artemisinin suppositories were found to be very effective and well tolerated in treating severe malaria in children (Phuong et al., 1997). Artemisinin suppositories were reported to be safe and more effective than IV quinine in treating complicated severe *falciparum* malaria in Ethiopian adults (Birku et al., 1999). Several other studies have also been done using artemisinin suppositories such as in Vietnamese patients with malaria (Arnold et al., 1990; Koopmans et al., 1998); in adults

with complicated and severe malaria in Vietnam (Ha et al., 1997). Dihydroartemisin suppositories were also reported to be effective in different studies such as in patients with severe non cerebral malaria (Falade et al., 2007); in Kenyan children and adults with severe malaria (Esamai et al., 2000); in patients with severe malaria in Thailand (Wilairatna et al., 2000).

Artesunate suppositories were found to be well tolerated and effective in Papua Guinea children (Karunajeewa et al., 2003). Artesunate suppositories were also tested in patients with severe malaria in Thailand (Looareesuwan et al., 1997); in Sudanese adults with severe *P. falciparam* malaria (Awad et al., 2003); in Vietnamese adults who had uncomplicated malaria (Benakis et al., 2006); in children with *P. falciparum* malaria in Gabon (Halpaap et al., 1998).

A comparison of the safety and efficacy of artemether suppogel and IV quinine also showed that artemether was well tolerated and effective in treating cerebral malaria. (Aceng et al., 2005). Various antimalarials have been formulated for rectal drug delivery such as artesunate suppositories i.e. Rectocaps® which are manufactured by Mepha Pharmaceuticals, Aesch–Basel, Switzerland (Persaud et al., 2020). Administration of a single dose of rectal artesunate (RAS) as a pre referral malaria therapy for children under the age of six years in rural regions of Sub-Saharan Africa when in suspicion of severe malaria was a recommendation by WHO in 2006 and has been beneficial in saving lives. RAS as a monotherapy was not recommended in uncomplicated malaria to decrease the risk of developing resistance (de Carvalho et al., 2021).

However, in severe malaria the administration of rectal artesunate suppositories (RAS) in the community or lower level of health care without referring children to the hospital or facility with high level of care for either IV administration or following up with an oral ACT once children start tolerating oral medicines is not beneficial (Watson et al., 2023). It has been reported that although the community access to rectal artesunate for malaria (CARAMAL) study which was an observational study about the deployment of RAS in Democratic Republic of Congo (DRC), Nigeria and Uganda, reported that RAS was linked to a rise in childhood deaths in Nigeria, the study design did not support a causal relationship between the deployment of RAS and a rise in deaths. The circumstances under which RAS can be helpful were not met in their settings (Hetzel et al., 2023). RAS is beneficial in severe malaria in children when treatment is given earlier with subsequent referral to the hospital or with a follow up treatment. The WHO MPAG currently does not recommend administration of RAS in severe malaria in rural areas when referral to the hospital is not possible or not efficient (Watson et al., 2023). Therefore, availability of a rectal ACT would offer more benefit than use of monotherapy in children who cannot tolerate medication or are unconscious as in cerebral malaria. Table 7 shows some examples of available rectal antimalarials. It can be noted that many of the products were formulated using only one drug and not the

two drugs as per WHO recommendation. The only product containing ACTs as recommended by who is a suppository that contains AR and LF by Galen Pharmaceuticals. *However, considering the very small rectal fluid volume (3 mL under normal conditions), the dissolution and absorption processes of these poorly soluble drugs when administered in suppository form could be affected* (Rathi et al., 2022). Hydrophilic bases need to dissolve first in the limited rectal fluid volume to release the drugs while lipophilic bases need to melt at body temperature to release the drugs. Therefore, delivering ACTs as a liquid dosage form bypasses the dissolution stages that suppository bases undergo as the drugs are already in solution.

Table 7. Examples of some available antimalarial rectal preparations

Drug & strength	Dosage form	Trade name	Formulation excipients	Manufacturer	Indication	Reference
Artesunate	Suppository (rectal capsule)	Rectocaps®		Mepha Pharmaceuticals, Aesch- Basel, Switzerland		(Persaud et al., 2020)
Artesunate 100 mg	Rectal artesunate suppository	RAS		Cipla	Prereferral treatment of severe malaria in children aged 6 months to 6 years old	mmv.org/newroom/news-resources-search/cipla
Artesunate 50 mg or 200 mg	Suppository	RESUNATE 50, RESUNATE 200	Suppository base	Galen Pharmaceuticals Limited (WHO-GMP certified)		[2]galenltd.com/artesunate-suppositories/
Artesunate 100 mg	Rectal capsule	ARTECAP 100 mg	lycerol, gelatine, titanium dioxide Medium chain triglyceride Hard fat	Strides Pharma Science Limited, India	Prereferral treatment of moderate or severe malaria in children aged 6 months to 6 years who are not able to take oral medication or get injectable medication	

Drug & strength	Dosage form	Trade name	Formulation excipients	Manufacturer	Indication	Reference
Artemether 40 mg	Suppogel (rectal capsule)	Artesiane® suppogel	Capsule shell: glycerol, gelatine, purified water Content of capsule: refined soybean oil	Dafra Pharma GmbH	Treatment of severe malaria and in pretransfer treatment in children, adolescents to elderly followed by a combination therapy Dose: 4 mg/kg body weight on the first day then 2 mg/kg body weight from day 2 when they cannot take oral medication	Dafra Pharma [1]SmPC
Artemether-lumefantrine 20/120mg, 40/240 mg and 80/480 mg		ARTEGAL-140, ARTEGAL-280 AND ARTEGAL-560	Suppository base	Galen Pharmaceuticals Limited (WHO-GMP certified)		[2]galenltd.com/artemether-lumefantrine-suppositories/#

[1]SmPC = summary of product characteristics. Accessed on 10th June 2023 at 23:00 h from dafrapharma.com/products/artesiane-suppogel/[2]Accessed on 11th June 2023 at 17:28 h

1.9 Statistically based design of experiment

The traditional approach when designing experiments is to change one process factor or component at a time (OFAT) in a formulation which may lead to interactions of factors being overlooked. In addition the traditional approach is time consuming and costly. The main advantage of utilizing statistically based design of experiments (DOE) is that all potential process factors or components and their possible interactions can be studied simultaneously at different levels (Ravi et al., 2014; Thorsteinsdóttir & Thorsteinsdóttir, 2021). The condition of the experiment is controlled by indepenedent variables known as factors (X) while the variables that can be measured as a result of experimental design are called responses (Y). The setting for a factor is known as level and must be at least one low (-1) and one high (+). The model is a description of the mathematical relationship betwwen X and Y. Linear, interaction and quadratic polynomial models are the major kinds of models. The main use of the linear and interaction models is for investigating the experimental system and are used together with screening or robustness test designs. A quadratic model which is able to detect curvature is chosen if the relationship between factors and responses is nonlinear. Inclusion of a quadratic term in the model enables determination of the optimal point (Thorsteinsdóttir & Thorsteinsdóttir, 2021).

During development of a formulation, DOE can help in determination of the most appropriate combination of formulation components (Maroju et al., 2018; Mhango et al., 2017). The main stages of DOE are: screening, optimization and robustness testing. Screening is done to investigate factors that may have an influence on the response(s) in order to find out significant factors and interactions. Optimization is done to find conditions that give optimal results. Robust testing is done to show absence of significant effect when validating the method. In other words to verify that operating ranges can work correctly with small changes in the variables (Aguilera et al., 2006; Yu et al., 2014). Various specialized softwares are available for designing and analyzing experiments (Ho et al., 2022; Ravi et al., 2014). MODDE® which means (**MOD**eling and **De**sign) is one of the specialized software products and has been used in this project. The main application kinds in MODDE® are screening, system characterization, optimization and robustness testing. When limited information is already available, system characterization can be used.

Various screening designs such as full factorial, fractional factorial, D-optimal and many more are used to find out the significant factors in a process. The full factorial design is a screening design that is able to identify main effects and interactions independent from each other. In addition, it is easy to understand and interpret, requires few runs per factor investigated, accommodates extra runs for failed experiments, can be easily upgraded to optimization designs such as composite design and is cost effective. However, its limitation is that it is only reasonable for experiments with only few factors (2-4 factors). The number of experiments increases with an increase in the number of

factors (Montgomery & St, 2022). The experimental domain of a full factorial design is represented as a cube as shown in Figure 2. A two level factorial design with two factors has 2^k dissimilar experiments, where k is number of factors. Such a design is also known as (2^3 design) and contains eight experiments. The dots show the corners of the design box and represents the order of experiments (Das & Dewanjee, 2018).

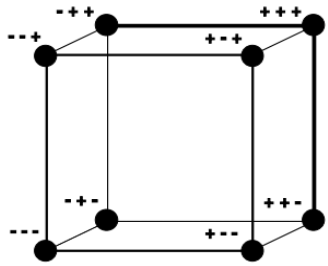

Figure 2. Representation of a full factorial design with three quantitative factors

The two level full factorial design can also be illustrated as shown in Appendix A.

The four main types of design of experiments are process experiments, mixture experiments, a combination of process and mixture experiments, and DOE with irregular experimental domains. However, only the design of process experiments will be explained briefly since it was used in this project. In design of process experiments, the response Y is a function of the amounts or levels of the factors. Alterations in amounts of each factor X_k are not linked to (= independent of) alterations in other factors. During analysis stage the model can be fitted to its design using different regression models such as multiple linear regression (MLR) or partial least squares (PLS). MLR describes the relationship or the strength of the relationship between two or more independent variables and a dependent variable by fitting a line to the observed data (Bartholomew, 2010; Peter et al., 2019). MLR is used in many fields including formulation development to predict or estimate how changes in two or more process factors affect a single response (Fridgeirsdottir et al., 2018; Herneisey et al., 2019).

After fitting the model to the design, it is important to know whether the design is good or not. Various parameters that can be used to judge the quality of a design are correlation matrices, condition number, degrees of freedom (DF), design power et cetera. Correlation matrix show how model terms are correlated and is represented by the value of the correlation coefficient r. Condition number is used to measure the sphericity of a design or simply show how well the experiments are spread. If the experiment fails, the DF for lack of fit is used to show how many extra runs are available and if the model can still be created.

During the analysis stage various functions can be used to fit and analyze data and can be classified into model group, residual analysis group and model interpretation group and many more. In the model group, the quality of the model can be checked using

summary of fit. The summary of fit plot is presented using R^2, Q^2, model validity and reproducibility. It is recommended for all bars to be close to 1 which also means 100 % (perfect) as shown in Figure 3.

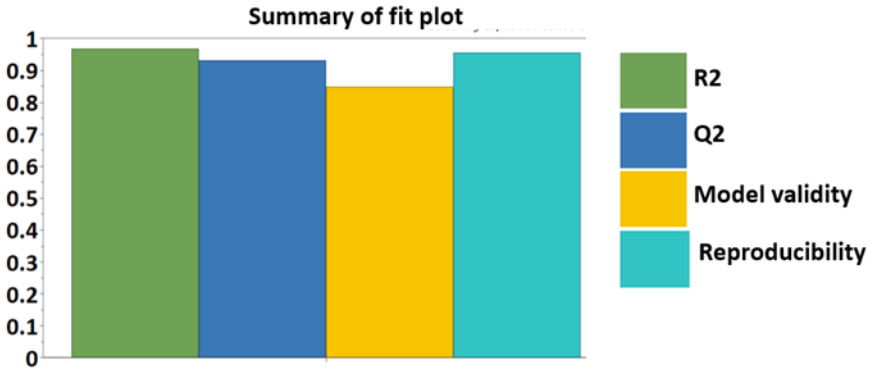

Figure 3. Summary of fit plot showing model quality

Model fit is shown by R^2 and a model with R^2 of 0.5 is a model of relatively little significance. Precision is important in experiments and formulation development as it shows the extent of agreement or closeness of two measurements to each other when measurements are repeated (Betz et al., 2011). The estimate of the future prediction precision is shown by Q^2 which is the best and most sensitive indicator, and its value should be larger than 0.1 and larger than 0.5 for a significant model and good model respectively. For a good model the difference between R^2 and Q^2 should be smaller than 0.3 or less than 20 % in almost all circumstances. The model can be improved by either choosing a suitable transformation in the histogram or excluding non-significant terms in the coefficient plots.

Validation is widely used in many fields including formulation development to measure the quality of research in terms of its accuracy (Andrade, 2018; Kumar et al., 2023). Model validity is used to test different model problems (to measure the validity of the model). A value greater than 0.25 indicates a valid model while a low value may indicate availability of outliers, statistically significant model problems, a wrong model, a transformation problem or missing of an interaction or square term. However, the presence of almost identical replicates (very small pure error) may make the value of the model validity to be very small even though the model is good and complete. In addition, the model validity is not calculated and is therefore labelled missing when the pure error is very small (replicates are almost identical). So, in very good models when Q^2 is greater than 0.9 due to very good replicates (high sensitivity) model validity may be low. Therefore, if model validity is very small, one need not to worry but to find out what is causing it to be low by checking warnings.

The degree to which consistent (reproducible) results are obtained upon repetition of the experiments is greatly required in many research areas even in formulation work (Roulon et al., 2021; Samsa & Samsa, 2019). Model reproducibility shows the replicates compared to the overall variability. Reproducibility should be larger than 0.5. Since in formulation development the aim is to develop formulations of high quality at a reduced cost, valid production methods need to be accurate, precise, and reproducible. Therefore, analysing the fit methods of the model is very important.

Further analysis of model quality is done using diagnostic plots such as residuals,

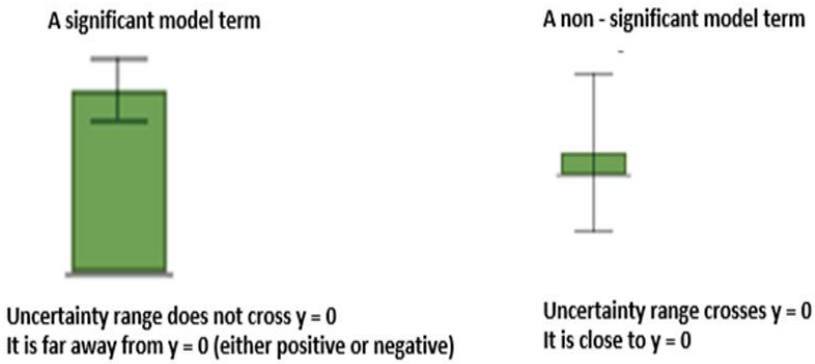

Figure 4. Graphical representaion of coefficient plots showing a significant and a nonsignificant model term

analysis of variance (ANOVA), and observed versus predicted plots et cetera (Castillo et al., 2019). Residual plots can be used to find outliers, drift, trends etc. in order to know how well the data is predicted. If the points follow close to a straight line on a residuals normal probability plot, they are said to be normally distributed and points that deviate from the probability line are called outliers. The residual plot of observed versus predicted plot should have points following a straight line for a good model. The use of residual plots is also reported in literature (Mohr et al., 2022). The ANOVA can be used to review the lack of fit. The lack of fit is estimated only when replicated points are available because it compares the pure error (replicate error) and model error (lack of fit). The influence of terms on the model can be interpreted using coefficient plots, effect plots, and lists. The coefficient plot is a graphical illustration of model terms for determining their significance as shown in Figure 4.

A very high (greater than 3000) condition number and absence of DF are among some of the reasons why confidence intervals are not shown on the coefficient plots. Graphical presentation of effect plots is similar to coefficient plots. However, the values of the effects for process factors are computed as twice the MLR coefficients, and are plotted sorted in absolute value in descending order. The error bars show the ± 95% confidence interval (CI) which can also be changed by user. The main effect plot for

process factors shows the values predicted for the chosen response when the factor changes over its range while the average value of all other factors in the design are kept constant. When one is satisfied with the model, then predictions and optimal conditions can be done using the model (Yu et al., 2014).

Optimizing formulation processes using DOE is common (Almotairi et al., 2022; Kumar et al., 2023; Ogbonna et al., 2017). When the conditions and objectives of responses have been specified, the minimum and maximum responses can be predicted. Then one can compare the specified responses with the predicted responses. When the difference between the desired range i.e. maximum and minimum and difference between predicted maximum and minimum is large, the possibility of finding a design space becomes great. It is important to remember that optimization is only done when significant factors have been identified (Kumar et al., 2023; Mhango et al., 2017).

The output of the model can be viewed using response contour plots, sweet spot plot, design space plot and desirability plots. Response contour plots show the values for the chosen responses. Regions where responses are within the user specified ranges are shown by the sweet spot plots. The colour scale from green to blue (green, blue, white and other colours) is used by the sweet spot plot but only the green colour shows the area where all responses are within the selected range. Contour plots have also been used by other researchers in formulation development to present their results (Almotairi et al., 2022).

The design space shows the best (optimal) set point which is represented by the circle. The robust set point can also be shown and is represented by a crosshair. In addition, the design space shows the probability of failure (%) for the displayed factor combination. The desirability plot is used to visualize or show how well the objective targets are attained within the design space. When all objectives are fulfilled the value, one is shown in the plot and zero is displayed when no objectives are attained. The greater the value, the nearer are all responses to their target value. The design space is vital when choosing a set point for which target values are attained as good as possible. The greatest desirability is not like the centre of the design space or to the most robust set point.

Considering the usefulness of DOE when conducting experiments, MODDE was used to design some experiments in formulation and HPLC work in this project.

2 Aims

The aim of the project was to design, evaluate and optimize a formulation that can be used in the pediatric population for the treatment of malaria, especially severe malaria. The project is divided into the following parts:

1. To develop a pediatric liquid formulation, containing a fixed dose combination of artemether and lumefantrine for rectal delivery (a rectal enema).
2. To evaluate the formulation in terms of the physical, chemical properties.
3. To conduct a bioequivalence study in rabbits, to evaluate the pharmacokinetics of the rectal formulation and compare rectal pharmacokinetics with a commercially available oral powder for suspension.

3 Materials and Methods

3.1 Materials

3.1.1 Materials for formulation work

Artemether and lumefantrine were kindly provided by Mangalam Drugs and Organics LTD. (India). Trifluoroacetic acid, soybean oil, polyethylene glycol 400, Glycofurol (tetraglycol) were bought from Sigma Aldrich (UK), formic acid was bought from Fluorochem (UK). Medium chain triglyceride (Labrafac Lipophile WL 1349) was kindly provided from Gattefossé SAS (France), Decanoic acid and monounsaturated omega-9 fatty acid (oleic acid) were bought from Tokyo Chemical Industry, (Belgium). Medium chain triglyceride (Miglyol® 812 N) was kindly provided from Oleochemical (IOI) Pharma GmBH (Germany). Methoxy polyethylene glycol 350 (Carbowax TM Sentry) was kindly provided from The Dow Chemical Company, (Midland, UK). Polysorbate 20 (Kolliphor® PS 20) was kindly provided from BASF (Berlin, Germany). Extra virgin olive oil (100 %) was bought from GEA (Slovenia), methanol and acetonitrile were purchased from Honeywell (Germany). The water was purified using a Milli-Q water purification system.

3.1.2 Materials for animal study

Lonart® 20/120 (Bliss GVS Pharma LTD, India) powder for oral suspension was provided by a local community pharmacy in Malawi. The rectal enema was prepared at the Faculty of Pharmaceutical Sciences and contained 8.7 mg artemether and 52 mg lumefantrine per mL. New Zealand White male and female rabbits were purchased from Granja San Bernardo (Spain). Eppendorf tubes (1.8 mL), 2 mL amber colored glass vials, 22G catheters, 2.5 mL syringes, normal saline (0.9% saline), alcohol 96%, were purchased from Proquilab (Murcia, Spain), heparin 1000 IU/mL and female catheters were purchased from Aleman Pharmacy (Murcia, Spain) weighing scales were purchased from Mettler Toledo (Barcelona, Spain), vortex and mini spin centrifuge were purchased from Merck Life Science (Madrid, Spain).

3.1.3 Materials for plasma sample analysis

Artemether, lumefantrine and lumefantrine-d9 were purchased from Santa Cruz Biotechnology, (Germany), Methanol and formic acid were purchased from Carl Roth (Denmark), acetic acid was purchased from Sigma Aldrich, well plates were purchased from Waters.

3.2 Methods

3.2.1 Preliminary screening (pilot study)

A pilot study was conducted to screen oily vehicles. The quantities used in the pilot study ranged from the minimum to the maximum doses recommended clinically for artemether while for lumefantrine quantities less than the minimum recommended dose were also used. Varying quantities of AR (20 mg – 60 mg) were added to 1 mL of different oil: medium chain triglyceride (Miglyol® 812 N), soybean oil, polyethylene glycol 400 and methoxy polyethylene glycol 350 in a 2 mL microtube. The mixtures were sonicated for 2 min at 50 % amplitude using Qsonica sonicators, Model CL-18, serial No. 2013061001, (Cole Parmar, UK) and stored at room temperature (21-23 °C). Varying quantities of LF (30 mg – 365 mg) were also dissolved in 1 mL of the respective vehicles in the same way AR was dissolved and stored at room temperature. In addition, known quantities of LF were dissolved under sonication in 1 mL of respective vehicles. After LF had dissolved, AR was also added and the mixture was sonicated again and kept at room temperature to observe if precipitation would occur or not.

3.2.2 Saturated solubility study

Saturated solubility study was then conducted on vehicles that showed potential in solubilizing the drugs. Various vehicles (1 mL) were put in a 2 mL Eppendorf tube and excess quantity of AR and LF were added in different tubes. Samples were mixed by vortexing on IKA® VORTEX 4 digital Model: PA1024-3I (Staufen, Germany) and thereafter allowed to shake at a speed of 250 min^{-1} for 24h on an Edmund Bühler GmbH shaker at room temperature (21-23 °C). After 24h samples were centrifuged at 5000 rpm for 10 min in a high-speed microcentrifuge (Corning LSE™, Vordingborg, Denmark). The supernatant was then diluted appropriately (500-fold dilution) with either ACN (i.e., for AR dissolved in MCTs and mPEG 350) or a mixture of ACN and MeOH for the drug that was dissolved in oleic acid. For LF dissolved in MCT and mPEG the supernatant was diluted 801-fold and 601-fold respectively using ACN while for oleic acid the dilution factor was 2401 using a mixture of ACN and MeOH. Acetonitrile was used as the blank and Genesys 150 UV VIS spectrophotometer (Thermo Fisher Scientific, Denmark) was used for quantifying lumefantrine at 336 nm (Sharma, 2016). The Reverse Phase High-performance liquid chromatography (RP-HPLC) system analyses were done using a Dionex Ultimate 3000 HPLC, HPG- 3400 pump with degasser, WPS 3000 TSL autosampler, TCC 3100 thermostated column compartment, Photodiode array (PDA) 3000 detector (Thermo Fisher Scientific, Denmark and Chromeleon chromatography workstation for the quantification of AR at 218 nm. A Phenomenex column Luna® 5µm C18(2) 100 Å, 250 x 4.6 mm with Guard column™ C18, 4x3 mm was used. The mobile phase for AR consisted of 0.1 % TFA in both ACN and milliQ (ACN: milliQ; 76:24 % v/v at a flow rate of 1.1 mL/min, at 25 °C. The

standard solutions and the samples were filtered using the Phenomenex 0.2 µm syringe filters before injection and the injection volume was 100 uL (full loop). The samples were run under isocratic mode. LF solubility in decanoic acid was done by melting the lipid at a temperature slightly higher than the melting point of the lipid and then adding the drug in small increments. The solubility of LF in the molten lipid was determined by visually observing the presence of undissolved drug.

3.2.3 Formulation preparation

Based on the results of the pilot study, MCT (Miglyol® 812 N), decanoic acid and monounsaturated omega-9 fatty acid (oleic acid) acid were selected for further investigation. The quantity of AR (20mg) and LF (120 mg) were held constant in all the formulations that were prepared. To prepare the formulation, the excipients were mixed and heated in a water bath till the fatty acid was fully melted. LF was then added to the lipid mixture on the water bath and was subjected to manual shaking occasionally, till it dissolved. Once LF dissolved, AR was added. After complete dissolution of the active pharmaceutical ingredients (APIs), the samples were stored at room temperature and were protected from light by wrapping the transparent glass bottles containing samples with aluminum foil. The composition of the monounsaturated omega-9 fatty acid-based formulation is shown in Table 8.

3.2.3.1 Monounsaturated omega-9 fatty acid based formulation

Table 8. Formulation composition of monounsaturated omega-9 fatty acid based formulation

Formulation components	% w/v, range
Artemether	1.1
Lumefantrine	6.67
Kolliphor PS 20	0.2
mPEG 350	4.47
Oleic acid	4.3 to 45.5
Miglyol® 812 N	45 to 86.9
Temperature	25 to 60 ±2 °C

3.2.3.2 Decanoic acid-based formulation: screening using design of experiment

MODDE® 13 from Sartorius (Sweden), was used to design the experiments to

investigate important factors that might have influenced the main results. A two-level full factorial design with three factors were put at two fixed points, (at minimum or high level). The interaction model comprised a total of 13 runs, incorporating five center points to assess the reproducibility of the method and model quality. Statistical analysis was done by the same software. The significance of the effect was calculated using the analysis of variance (ANOVA). The default confidence level (95 %) of MODDE® was used. The regression relationship between factors and responses was done using the F - test. The factors that were used in DOE are shown in Table 9 and formulation composition is shown in Table 10. Limitations on minimum values to be used were placed on decanoic acid amount and temperature based on preliminary experiments.

Table 9. Factors and settings used in MODDE for the decanoic acid based formulation

Name	Type	Settings
Decanoic acid amount (g)	Quantitative	0.1 to 1
Miglyol® 812N amount (g)	Quantitative	1 to 2
Temperature	Quantitative	35 to 60 ± 2 °C

Table 10. Formulation composition of the decanoic acid based formulation

Formulation components	% w/v, range
Artemether	0.87
Lumefantrine	5.22
Kolliphor PS 20	0.2
mPEG 350	4.47
Decanoic acid	4.3 to 45.5
Miglyol 812N	45.5 to 86.9
Temperature	35 to 60 ±2 °C

3.2.3.3 Optimization of the decanoic acid-based formulation using optimizer tool in MODDE

The optimizer tool was used to find the sweet spot area, design space, desirability and robust set point. The factors and roles that were used are shown in Table 11 and the objectives and conditions are shown in Table 12.

Table 11. Factors and roles used for the optimization of decanoic acid based formulation using DOE

Factor	Role	Low limit	High limit
Decanoic acid amount (g)	Free	0.1	1
Miglyol® 812 N (g)	Free	1	2
Temperature (°C)	Free	35	60

Table 12. Objectives and conditions for optimization of decanoic acid based formulation using DOE

Objectives and conditions for lumefantrine precipitation time (months)		
Response	**Objective**	**Condition**
LF precipitation	Maximize	Required
LF dissolution	Observed	Predicted
Objectives and conditions for lumefantrine dissolution time (min)		
LF dissolution	Required	Minimize
LF precipitation	Observed	predicted

3.2.3.4 Formulation composition containing 10 % of a mixture of monounsaturated omega-9 fatty acid and decanoic acid

These formulations were prepared without using DOE. The formulations consisted of a mixture of decanoic acid and monounsaturated omega-9 fatty acid (10 %) of the total solvent system. The quantity of decanoic acid, monounsaturated omega-9 fatty acid and Kolliphor® PS 20 varied while that of AR and LF and temperature were held constant. This formulation did not contain mPEG 350. The different proportions of decanoic acid and monounsaturated omega-9 fatty acid are shown in Table 13 and the actual formulation composition is shown in Table 14.

Table 13. Formulation based on a mixture of decanoic acid and monounsaturated omega-9 fatty acid (10 %) and the percentage of each fatty acid in the formulation

Excipient	**Formulation**		
	F 1	F 2	F 3
Monounsaturated omega-9 fatty acid (%)	25	50	75
Decanoic acid (%)	75	50	25

Table 14. Formulation composition based on a mixture of decanoic acid and monounsaturated omega-9 fatty acid (10%)

Formulation components	**Formulation (%)**					
	F 1	F 2	F 3	F 4	F 5	F 6
Artemether	X	X	X	X	X	X
Lumefantrine	Y	Y	Y	Y	Y	Y
Miglyol® 812 N	89.8	89.5	89.8	89.5	89.8	89.5
OA/DAC 1	10	10	0	0	0	0
OA/DAC 2	0	0	10	10	0	0
OA/DAC 3	0	0	0	0	10	10
Kolliphor® PS 20	0.2	0.5	0.2	0.5	0.2	0.5
Temperature (°C)	60	60	60	60	60	60

3.2.3.5 Formulation containing a mixture of decanoic and monounsaturated omega-9 fatty acid in a 1:1 ratio

Another formulation was prepared comprising of a mixture of decanoic acid and monounsaturated omega-9 fatty acid in a 1:1 ratio. The total contribution of decanoic acid and monounsaturated omega-9 fatty acid mixture in the solvent system was 1%, 5%, 10%, and 15%. The formulations did not contain mPEG 350 as shown in Table 15.

Table 15. Formulation composition based on on a mixture of decanoic acid and monounsaturated omega-9 fatty acid contributing 1%, 5%, 10%, and 15% in the total solvent system.

Formulation components	**Formulation**			
	F 1	F 2	F 3	F 4
Artemether (g)	X	X	X	X
Lumefantrine (g)	Y	Y	Y	Y
Miglyol® 812 N (%)	99	95	90	85
OA/DAC (50:50) %	1	5	10	15
Kolliphor® PS 20 (%)	0.5	0.2	0.2 & 0.5	0.2 & 0.5
Temperature (°C)	60	60	60	60

3.2.3.6 Formulation containing varying ratios of mPEG 350, decanoic acid, and MCT

The formulation in Table 16 consisted of varying ratios of all mPEG 350, decanoic acid and MCT. The quantities of AR and LF were held constant.

Table 16. Factors used for the formulation containing varying quantities of mPEG 350, decanoic acid and Miglyol® 812N

Formulation excipients	Settings
mPEG amount (g)	0.12 to 1.75
Decanoic acid (g)	0.02 to 0.46
Miglyol® 812N (g)	0.12 to 1.75
Temperature (°C)	35 to 60 ± 2 °C

3.2.4 HPLC method development for quantification of artemether and lumefantrine in lipid vehicles

3.2.4.1 Determination of lambda max of artemether and lumefantrine

During analysis wavelengths where APIs have their absorbance maxima should be used to get maximum sensitivity and the best linear calibration curve. Therefore, the initial step was to determine the lambda max of AR and LF according to the method reported in the International Pharmacopoeia (Ph. Int.) 9th edition. AR and LF were dissolved in methanol by sonication to make concentrations of 10 mg/mL and 0.1 mg/mL respectively. After sonication, the solutions were allowed to cool to room temperature and diluted 5-fold (i.e. 1 mL of the solution was diluted with 4 mL methanol). This was followed by scanning from 190 nm to 400 nm and 275 to 325 nm for AR and LF respectively. LF was also scanned from 190 to 400 nm. An amber colored volumetric flask was used when dissolving AR while for LF, a transparent flask was used. Quartz cuvettes (pathlength 10 mm, Thermo electron Starna Scientific LTD) and the Genesys 150 UV-VIS spectrophotometer were used. The Quartz cuvettes were rinsed with methanol and left to dry before using them. Methanol was used as the blank during the analysis.

3.2.4.2 HPLC quantification method

The initial plan was to use a modified simple HPLC assay method described for AR intramuscular injection in the International Pharmacopoeia, 9th edition from literature. A reverse phase high performance liquid chromatography system from Dionex instrument was used. Details of this instrument have already been stated under the section (saturated solubility study). The Phenomenex columns: Luna® 5 µm C18(2) 100Å 250 x 4.6 mm attached to a guard column (Guard column™ C18, 4 x 3 mm SecurityGuard™ part No: AJO-4287) and a Kinetex 5 µm C8 100 Å, 150 x 4.6 mm attached to a SecurityGuard™ ULTRA HOLDER (part No: AJO-9000 and SecurityGuard™ ULTRA cartridges, UHPLC C8 4.6 mm ID (part No: AJO-8770) were used. Various experiments

were conducted to find a simple analysis method that can give good retention of the APIs and separation between APIs as well as from excipients to avoid interference caused by lipid excipients when quantifying AR and LF. Initial experiments were conducted without using DEO and the later and many experiments were done using MODDE®.

Mobile phase preparation

The mobile phases that were tried consisted of different proportions of acidic water and acidified organic modifiers (either MeOH or ACN). Formic acid and trifluoroacetic acid (TFA) were used to adjust the pH of the mobile phases. The mobile phases contained 0.1 % of the stated acids. The prepared mobile phases were degassed by vacuum filtration using a 0.45 µm nylon filter followed by sonication in a bath sonicator. Before analysis the mobile phases were degassed again using an online degasser of the instrument and the HPLC lines were also cleaned.

Preparation of stock solutions

AR and LF stock solutions were prepared by dissolving them in methanol by sonication in separate volumetric flasks to make concentrations of 10 mg/mL and 0.1 mg/mL for AR and LF respectively. The amber-colored flask was used when dissolving AR alone as well as when AR and LF were dissolved in the same flask. For dissolving LF alone, a transparent flask was used. After the APIs had dissolved, the volume was made up to the required volume with MeOH.

Preparation of standard solutions

The standards were prepared by diluting the stock solutions with the mobile phase to the required concentrations of 5 to 3000 µg/mL and 0.05 to 30 µg/mL for AR and LF respectively. The standards were filtered using the Phenex™ 4mm regenerated cellulose membrane, 0.2 µm, 4 mm syringe filters before injection and the verex vial, snap, 2 mL, amber colored vials were used. The injection volumes, 20 µL and 100 µL were used. Calibration curves were prepared and concentration versus peak area was plotted for AR and LF. The obtained data was subjected to regression analysis. Analysis was done in triplicates. In addition, the most concentrated standard (n=6) was prepared and analyzed on different days. Accuracy was measured as percentage recovery from the prepared formulations prepared either same day or in less than 48 hours after appropriate dilutions.

Preparation of formulations for analysis

Oily formulations/samples for analysis were diluted mainly with ACN or MeOH or a mixture of ACN and MeOH when they contained monounsaturated omega-9 fatty acid as one of the excipients. Very concentrated samples were used for the analysis of AR.

Isocratic elution using the Luna® 5 µm C18(2) column 100Å 250 x 4.6 mm and 0.1 % TFA in methanol as the organic modifier

Different proportions of acidic MeOH and acidic water were tried. Table 17 shows the experiments that were conducted without using DOE and experiments in Table 18 were designed by MODDE.

Table 17. HPLC isocratic elution method based on 0.1% TFA in MeOH as an organic modifer without using DOE

Experiment	Acidic MeoH (%)	Flow rate (mL/min)	Temperature (±2 °C)	Wavelength (nm)
1	65	0.8	25	218, 277 & 336
2	65	1.3	25	218, 277 & 336
3	70	0.8	25	218, 277 & 336
4	70	1.3	25	218, 277 & 336
5	80	0.8	25	218, 277 & 336
6	80	1.3	25	218, 277 & 336
7	90	0.8	25	218, 277 & 336
8	90	1.3	25	218, 277 & 336

Table 18. Factors used in MODDE® using the Luna® 5 µm C18(2) 100 Å 250 x 4.6 mm column and 0.1 % TFA in methanol and an isocratic elution method

Factor	Type	Settings
Flow rate	Quantitative	0.8 to 1.3
0.1 % TFA in Methanol (%)	Quantitative	80 to 90
Wavelength (nm)	Quantitative	218 to 236
Temperature (°C)	Quantitative	25 to 40

HPLC Isocratic elution method comparing the Luna® 5 µm C18(2) and Kinetex 5 µm C8 using 0.1 % formic acid in acetonitrile as the organic modifier

The two Phenomenex columns: Luna® 5 µm C18(2) column 100Å 250 x 4.6 mm attached to a guard column (Guard column™ C18, 4 x 3 mm SecurityGuard™ part No: AJO-4287) and a Kinetex 5 µm C8 100 Å, 150 x 4.6 mm attached to a SecurityGuard™ ULTRA HOLDER (part No: AJO-9000 and SecurityGuard™ ULTRA catridges, UHPLC C8 4.6 mm ID (part No: AJO-8770) were used to find out which column was better at retaining the APIs as well as achieving separation between the APIs themselves and between the APIs and excipients. MODDE® was used to design experiments and factors and settings that were used are shown in Table 19. Columns 1 and 2 stand for the Luna® 5 µm C18(2) column and the Kinetex 5 µm C8 column respectively.

Table 19. Factors and settings used in MODDE® using the Lunar® 5 µm C18(2) and Kinetex 5 µm C8 columns.

Factor	Type	Settings
Organic modifier (0.1 % formic acid in ACN) %	Quantitative	50 to 80
Flow rate (mL/min)	Quantitative	0.8 to 1.3
Wavelength (nm)	Quantitative	218 to 254
Column	Multilevel	1 and 2

Gradient elution method using the Kinetex® 5 µm C8 columns and 0.1% formic acid in ACN as the organic modifier

Based on the results after testing the two columns, four gradient elution methods were tested using the Kinetex® column only. The mobile phases consisted of 0.1 % formic acid in ACN and 0.1% formic acid in water and run at a flow rate of 1.3 mL/min. Stock solutions were diluted with acidic ACN and acidic water (50:50 % v/v) and 20 µL was injected at 25 °C at 218 nm, 236 nm and 254 nm. Appendices B to D show the gradient elution methods that were tested.

Isocratic elution method using 0.1 % TFA in ACN as the organic modifier and the Luna® 5 µm C18(2) column

Following the results of the gradient elution method using the Kinetex column, an isocratic elution method that could find out the best conditions that can separate the APIs and excipients using the same column was developed by MODDE. Factors and settings that were used are shown in Table 20.

Table 20. Factors used in MODDE® to find a method that avoids excipient interference during analysis using the Luna® 5 µm C18(2) 100 Å 250x4.6 mm column and a Pre column and 0.1% TFA in acetonitrile as the organic modifier

Factor	Type	Settings
Organic modifier (0.1 % TFA in acetonitrile) %	Quantitative	65 to 90
Temperature (°C)	Quantitative	25 to 40
Wavelength (nm)	Quantitative	218 to 236
Flow rate (mL/min)	Quantitative	0.8 to 1.3

Optimization of the method using the Luna® 5 µm C18(2) 100 Å 250x4.6 mm column and a Pre column and 0.1% TFA in ACN as the organic modifier

The optimizer toolbox of MODDE was activated to analyze the optimal conditions or best compromise for separating the APIs and excipients after objectives and conditions of responses and roles of factors were specified. Several alternative runs were predicted but only the best runs with the lowest probability of failure are shown on the tables. Table 21 shows the factors and their roles and Table 22 shows responses and conditions that were specified and the predicted run when all the roles in Table 21 were selected to be free i.e. selecting from a range of the minimum to the maximum value of the quantitative factors.

Table 21. Factors and roles that were specfied in optimizer

Factor	Role	Low limit	High limit
ACN (%)	Free	65	90
Temperature (°C)	Free	25	40
WL (nm)	Free	218	336
FR (mL/min)	Free	0.8	1.3

Table 22. Conditions and objectives for the responses when all the roles were specified to be free

Response	Condition	Objective	Minimum
LF retention (min)	Observed	Predicted	
AR retention time (min)	Observed	Predicted	
LF separation time (min)	Required	Maximize	2
AR separation time (min)	Required	Maximize	1
AR-LF separation time (min)	Observed	Predicted	

Table 23 shows the factors and roles and the recommended conditions for the experiment when the value of ACN was chosen to be constant and Table 24 shows the conditions and objectives that were specified for responses and the predictions that were made by the optimizer tool.

Table 23. Factors and roles that were specified and ACN % is made constant

Factor	Role	Low imit	High limit
ACN (%)	Constant		
Temperature (°C)	Free	25	40
WL (nm)	Free	218	336
FR (mL/min)	Free	0.8	1.3

Table 24. Conditions and objectives for the responses and the predictions made when ACN% was specified to be constant

Response	Condition	Objective	Minimum
LF retention (min)	Observed	Predicted	
AR retention time (min)	Observed	Predicted	
LF separation time (min)	Required	Maximize	2
AR separation time (min)	Required	Maximize	1
AR-LF separation time (min)	Observed	Predicted	

Table 25 shows factors and roles and the recommended conditions for the experiment when both the FR and ACN % were specified to be constant and Table 26 shows the conditions and objectives that were specified for the responses and the predictions that were made by the optimizer tool.

Table 25. Factors and roles that were specified, ACN % and FR made constant

Factor	Role	Low limit	High limit
ACN (%)	Constant		
Temperature (°C)	Free	25	40
WL (nm)	Free	218	336
FR (mL/min)	Constant		

Table 26. Conditions and objectives for the responses and when both ACN % and FR were made constant

Response	Condition	Objective	Minimum
LF retention (min)	Observed	Predicted	
AR retention time (min)	Observed	Predicted	
LF separation time (min)	Required	Maximize	2
AR separation time (min)	Required	Maximize	1
AR-LF separation time (min)	Observed	Predicted	

In addition to using the optimizer tool, the contour plots were also used to find the possible setpoint or conditions under which separation can occur between APIs and excipients.

3.2.5 NMR analysis

NMR samples of the pure compounds of lumefantrine, artemether and medium chain triglycerides (MCT); as well as of the precipitates, both from lumefantrine alone in MCT, and from a mixture of lumefantrine and artemether in MCT, were prepared in deuterated chloroform ($CDCl_3$) and their 1H spectra were recorded on a Bruker Avance 400 MHz spectrometer and analyzed using MestReNova software.

3.2.6 Preclinical study in rabbits

3.2.6.1 Study design

An open randomized, three period, three sequence, single dose cross over study was performed at the Department of Pharmacology, Faculty of Veterinary Medicine at the University of Murcia, Spain between the months of May to July 2022. The study was approved by the Bioethical Committee of the University of Murcia, Spain (approved experimental protocol number (os 811/2022) and was carried out according to the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals. All efforts were undertaken according to the "3R principles" to reduce the number of animals used in the study and optimize experimental protocols for obtaining maximum

data from each tested animal. A limited number of rabbits were used since this was a pilot study to investigate LF absorption from the rectal solution.

A total of six healthy (four non pregnant female and two male) New Zealand White (NZW) rabbits weighing between 3.75 to 5.14 kg were housed in individual cages with free access to food and water *at libitum* and maintained on a 12/12 h light/dark cycle at controlled and fixed temperature and humidity at the laboratory animal facility at the Faculty of Veterinary Medicine at the University of Murcia, Spain. Before experiments animals were allowed to acclimatize for several days. In addition, they were weighed on a VETMAT 1020 of capacity 20 kg x 10 g scale and randomized into three groups.

Each animal was assigned both the microchip ID and a letter code. Before receiving treatments, the animals were weighed again. Table 27 shows the weight of animals during the experiments and Table 28 shows the study design. Figure 5 shows the cages in which the rabbits were housed during the experiments.

Table 27. The weight of rabbits during experiments

	Weight (kg)		
Rabbit ID	**Experiment 1**	**Experiment 2**	**Experiment 3**
A	4.72	4.75	4.88
B	4.14	4.12	4.14
C	4.73	4.68	4.76
D	3.75	4.08	4.14
E	4.60	5.01	5.04
F	5.00	5.01	5.14

Table 28. Rabbit allocation and experimental protocol

Rabbit ID	**Gender**	**Experiment 1**	**Experiment 2**	**Experiment 3**
A	Male	Oral nonfasted	Oral nonfasted	Oral fasted
B	Male	Rectal	Rectal	Oral nonfasted
C	Female	Rectal	Rectal	Oral fasted
D	Female	Oral fasted	Oral fasted	Oral nonfasted
E	Female	Oral nonfasted	Oral nonfasted	Rectal
F	Female	Rectal	Rectal	Rectal (repeat)

Figure 5. A rabbit housed in an individual cage during experiments

In each experiment animals were randomized to receive either (a) the oral suspension under fasted state, (b) or oral formulation under fed state; (c) or the rectal formulation. The animals that received the oral formulation under fasted state were starved 12 h before the study but had access to water. Animals were given 24 mg/kg of lumefantrine as a single dose. The rectal enema was administered 6 cm into the anus of the rabbit. A two-week washout period was allowed to elapse between treatments. Animals were observed daily for any possible adverse events. Figures 6 and 7 show the formulations that were administered to rabbits and the animal laboratory at the faculty of veterinary medicine respectively.

Figure 6. The oral suspension and rectal solution (enema) that were administered to rabbits

Figure 7. The animal laboratory at the faculty of veterinary medicine, Murcia in Spain

3.2.6.2 Blood sampling

The hair on ears of rabbits was shaved using an electric shaver and the shaved areas cleaned with 96% alcohol. Syringes were rinsed/flushed with heparin before blood collection to avoid blood coagulation during blood collection. A 22 G catheter attached to a 2.5 mL syringe was used to collect blood samples from the marginal ear vein of each rabbit before and after dose administration at the following time intervals: 0, 30, 45, 60 and 90 min, then after 2, 4, 6, 8, 10, 24, 48 and 96 h. Syringes (2.5 mL) flushed/rinsed with approximately 2 mL heparin (1000 IU/mL) were used to collect blood samples into appropriately labelled 1.8 mL transparent Eppendorf tubes. Figure 8 shows the rabbit during blood sampling.

Figure 8. The rabbit during blood sampling

Blood samples were immediately centrifuged on a mini spin Eppendorf at 12,000 rpm for 10 min. Plasma was then collected and divided into two amber colored glass vials (2 mL) and put in a freezer at -80 ± 3 °C. After blood was withdrawn, a few microliters of 20 IU heparin in normal saline was injected into the catheter to prevent blood coagulation in the catheter when attached to the ear of the rabbits. Animals that were fasted during the experiment were given access to food after collecting blood samples at 2 h. At the end of the study, some samples were packed and shipped to Iceland for analysis, *using reusable iceless containers* provided by World Courier shipping

company while the other samples were left in a -80 ±3 °C at the department of Pharmacology, University of Murcia in Spain. Upon arrival in Iceland the samples were stored in a freezer at -80 ± 3 °C until analysis.

3.2.6.3 Preparation of standard solutions

Stock solution of LF was prepared in MeOH with 0.5% formic acid. The internal standard (IS), Lumefantrine-d9 was dissolved in MeOH with 0.5% formic acid. All stock solutions were stored at -20°C until use. Working solutions containing LF were prepared using serial dilution with MeOH: water (50:50 v/v). Eight calibration samples and three quality control (QC) samples were prepared by spiking blank human plasma with 10 µL of appropriate working solution to 90 µL of blank plasma to create standards and quality control samples. The concentration of the calibration samples in plasma was 5, 10, 20, 50, 250, 500, 2000 and 5000 ng/mL for LF. QC samples in plasma were 15, 150 and 3000 ng/mL.

3.2.6.4 Rabbit plasma sample analysis

Rabbit plasma sample (100 µL) was added to a 96 well plate followed by 300 µL of MeOH + 0.5% acetic acid containing IS, plate was sealed with cap mat and samples vortexed for 1 min at 1,050 RPM. Samples were then centrifuged at 2,500 g for 10 min. The supernatant (200 µL) was injected into the UPLC - MS/MS system.

3.2.6.5 UPLC - MS/MS method for quantification of lumefantrine

The quantification of lumefantrine was performed on a Waters Acquity I-Class UPLC system, coupled to a Waters Xevo TQ-XS triple quadrupole mass spectrometer equipped with electrospray ionization (ESI) probe. Nitrogen was used as a desolvation and cone gas and high purity argon as a collision gas. Source temperature was set to 150°C and desolvation gas temperature was 600°C, at a flow rate of 1000 L/h; cone gas flow rate 150 L /h. The analytical column, Acquity Premier BEH (1.7 µm, 50 × 2.1 mm) (Waters Corporation, Wexford, Ireland), was maintained at 30°C. The injection volume was 2.0 µL and the sample manager temperature was maintained at ambient temperature. The gradient system consisted of mobile phase A: 0.5% formic acid in water and mobile phase B: 0.5% formic acid in methanol, at a flow rate of 0.50 ml/min. Initial conditions starting at 32% mobile phase A followed by a linear gradient to 88% of mobile phase B at 2.0 min then back to initial conditions at 2.1 min and then held for 1.9 min. The total chromatographic run time was 4.0 min and the retention time of lumefantrine was 1.6 min respectively. The mass spectrometer was optimized for analyzing lumefantrine and lumefantrine-d9 using multiple-reactions monitoring in the positive ESI mode to monitor precursor ion → product ion (m/z) at the transitions m/z 527.95 → 509.92 for lumefantrine and 538.90 → 521.03 for lumefantrine-d9. Lumefantrine-d9 was used as an internal standard for lumefantrine.

3.2.7 Estimation of pediatric dosage of antimalarial drugs, using pharmacokinetic and physiological approach

This part of the work was a theoretical estimation of dosage, based on physiological differences between children and adults. Several traditional methods of scaling dosages from adults to children that have been reported in literature were used to calculate pediatric doses of AR and LF. The following equations were used and published in paper 2:

Young's rule: $D_{child} = average\ adult\ dose\ \left(\frac{age\ of\ child(y)}{age\ of\ child\ (y) + 12}\right)$ (Eq. 1)

where D_{child} is the dosage in a child.

Clark's rule: $D_{child} = adult\ dose\ \left(\frac{weight\ of\ child\ (kg)}{68\ kg}\right)$ (Eq. 2)

Area rule: $D_{child} = adult\ dose\ \left(\frac{BSA\ of\ child\ (m^2)}{1.73\ m^{2*)}}\right)$ (Eq. 3)

Here, BSA was calculated according to Mosteller formula, where W is the weight in kg and H is the height in cm:

$$BSA = \sqrt{H \times \frac{W}{3600}} \quad \text{(Eq. 4)}$$

In addition, data on the PK of AR and LF (Karbwang et al., 1997), enzyme maturity and physiology in children that were obtained from literature were used (Bonate et al., 2018; Johnson et al., 2006). Doses based on the p data and the desired plasma concentrations were calculated using the following equations and the information in Table 29.

$$C_p = \frac{FDk_a}{V_d(k_a - k_e)}\left(e^{-k_e(t-t_0)} - e^{-k_a(t-t_0)}\right) \quad \text{(Eq. 5)}$$

Where F is the fraction of dose absorbed, V_d is the volume of distribution, K_a and K_e are the absorption rate constant and the elimination rate constant, respectively and t is the time of sampling, and t_o is the lag time. Then if the C_P found in adults is used and the PK parameters found in children (marked', with such as F') are added, the appropriate dose in children can be estimated.

$$D_{child} = \frac{C_p V_d'(k_a' - k_e')}{F' k_a'\left(e^{-k_e' t} - e^{-k_a' t}\right)} \quad \text{(Eq. 6)}$$

Table 29. Pharmacokinetic parameters of artemether and lumefantrine based on values from WHO and literature Karbwang et al., (1997); Lefèvre et al., (2002)

	Artemether		Lumefantrine	
Parameter	**Adult**	**Children**	**Adult**	**Chivreldren**
Dose (mg/kg)	1.14	5	6.86	29
C_{max} (ng/mL)	100	119	6.757	
t_{max} (h)	2		6	
$t_{1/2}$ (h)	1.9	16	95	123
AUC (ng.h/mL)	320	392	207	
V_d (L/kg)	6.05	0.9	3.80	
Cl(L/h/kg)	0.91			
Ka (/h)	1.52	1.2	0.44	0.46
F (%)	43		65	
Fu (%)	4.6		0.3	

AUC = area under the curve, C_{max} = maximum concentration, Cl = clearance, t_{max} = time to maximum plasma concentration, $t_{1/2}$ = half-life, F = bioavailability, Fu = unbound fraction, K_a = absorption rate constant, V_d = volume of distribution.

The maturation of main enzymes responsible for metabolizing AR (CYP2B6 and CYP3A4) and LF (CYP3A4) in children was calculated using the following equations (Johnson et al., 2006):

$$CYP2B6 = \frac{1.07 \times Age}{1.13 + Age} \quad \text{(Eq. 7)}$$

For CYP3A4, the rate of maturation changes around 2.3 years, so for children below the age of 2.3 years, the following equation was used (Bonate et al., 2018)

$$CYP3A4 = 0.11 + \left(\frac{0{,}95\ x\ AGE^{1.91}}{0.64^{1.91} + AGE^{1.91}}\right) \quad \text{(Eq. 8)}$$

For children aged over 2.3 years old, the rate of maturation was calculated using (Bonate et al., 2018):

$$CYP3A4 = 1.1 - 0.123 \times e^{(-0.05(AGE - 2.2))} \quad \text{(Eq. 9)}$$

The information from the enzyme maturation calculations was used further in calculating the overall degree of enzyme maturation (MFA) in children aged below 25 years by adding the calculation results in the following equation (Bonate et al., 2018; Templeton et al., 2018):

$$MFA = (0.111 \times CYP2B6) + (0.889 \times CYP3A4) \quad \text{(Eq. 10)}$$

The MFA information was then used to estimate the clearance of AR and LF in children below the age of five years using the following equation (Bonate et al., 2018):

$$Cl_{children} = Cl_{adults}\left(\frac{Weight}{70\ kg}\right)^{0.75} \times MFA \quad \text{(Eq. 11)}$$

It is important to note that this equation does not take into consideration the variations in the volumes of distribution in children compared to that in adults.

Standard liver volume (SLV) was calculated following Urata's equation (Walter et al., 2010):

$$SLV(mL) = 706.2\ X\ BSA(m)^2 + 2.4 \quad \text{(Eq. 12)}$$

Where BSA is body surface area. Urata refined equation 12 with respect to calculation of BSA for children that weigh below 15 kg and therefore used the equation that was proposed by (Haycock et al., 1978; Walter et al., 2010). Therefore equation 13 was only used to calculate BSA when calculating SLV in this project.

$$BSA(m^2) = BW(kg)^{0.378} X\ BH(cm)^{0.3964}\ X\ 0.024265 \quad \text{(Eq. 13)}$$

Where BH is the body height and BW is body weight.

4 Results

4.1 Preliminary screening (pilot study)

Solubilization of AR was observed in all the vehicles that were tested. LF, however, was very difficult to dissolve and it precipitated in all the vehicles that were tested. In samples that contained both AR and LF, precipitation started shorlty after sonication. Interestingly, precipitated LF dissolved when decanoic acid was added to the samples as shown in Table 30.

Table 30. Preliminary solubility of artemether and lumefantrine in various vehicles at 21 to 23 °C. The table was published in paper 1.

Drug & vehicle	Drug concentration (mg/mL)	Dissolved (Y/N)	Precipitation time
Artemether			
MCT	60	Yes	NP
Soybean oil	60	Yes	NP
mPEG 350*	62	Yes	NP
PEG 400**	60	Yes	NP
Lumefantrine			
MCT	30	Yes	1 hour
MCT	364	No	
Soybean oil	123	Yes	20 min
Soybean oil	248	No	
mPEG 350*	125	No	
Tetraglycol	90	No	
PEG 400**	73	No	
Olive oil	50	Yes	20 min

Drug & vehicle	Drug concentration (mg/mL)	Dissolved (Y/N)	Precipitation time
AR & LF			
MCT	5/30	Yes	24 h
Miglyol® 812 N	20/120	Yes	20 min
MCT + oleic acid	20/120	Yes	NP
MCT + decanoic acid	20/120	Yes	NP
Soybean oil	20/120	No	

*Methoxy polyethylene glycol 350; **Polyethylene glycol 400; NP = no precipitation

4.2 Saturated solubility in selected lipids

Selected lipophilic vehicles including MCTs, monounsaturated omega-9 fatty acid, decanoic acid (DCA), and mPEG were screened for further studies to identify potential lipids that could solubilize both drugs and keep them in solution. Figure 9 shows that the solubility of AR was good in all tested vehicles. LF, however, showed highest solubility in monounsaturated omega-9 fatty acid and lowest in mPEG 350. Generally, the solubility of AR in all the tested lipids was higher than that of LF. In solid lipids, LF was found to be dissolved in 30 mg/g in decanoic acid at 60 °C.

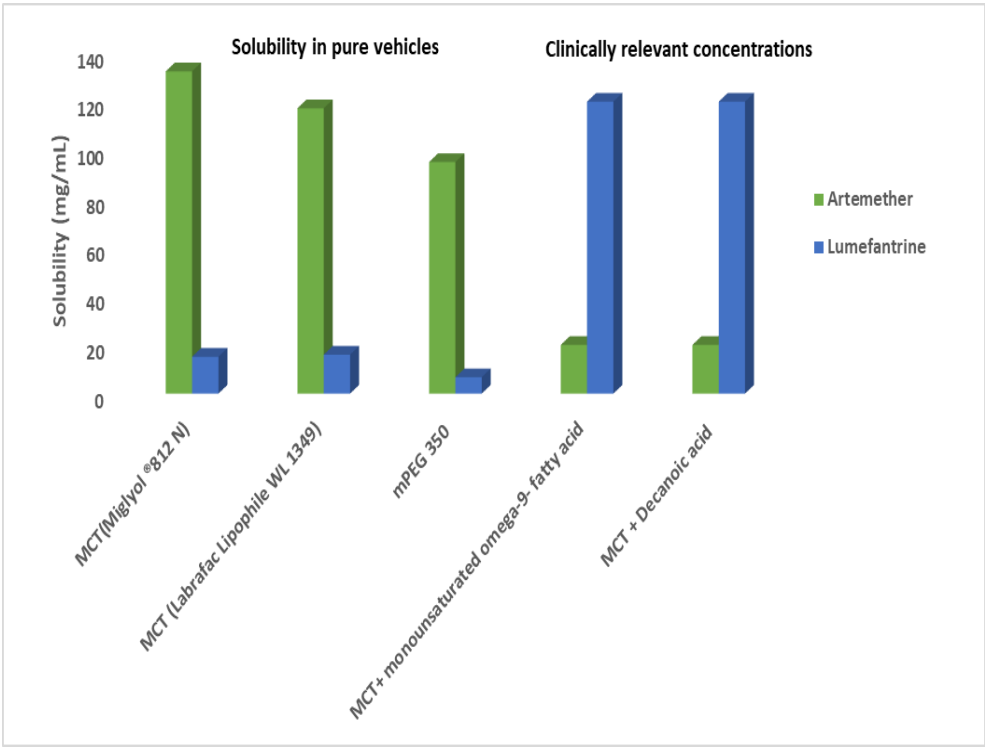

Figure 9. Saturated solubility of artemether and lumefantrine in selected pure vehicles and solubility in a mixture of pure vehicles and the fatty acid. The Figure was published in paper 1.

4.3 Incompatibility between artemether and lumefantrine

When LF and AR were mixed in the same formulation, i.e., MCT in the absence of decanoic acid, precipitation occurred. NMR analysis was performed on LF by itself (Figure 10a), the precipitate of an AR/LF mixture in MCT (Figure 10b), AR by itself (Figure 10c), as well as MCT by itself (Figure 10d). When the NMR spectrum of LF by itself was compared with the precipitate from the AR/LF mixture, it could be seen that most of the precipitate was LF, but there was a small peak at 3.43 ppm, indicating the presence of some AR. Other AR peaks were either too small to be observed in the NMR or hidden behind other peaks from LF. Additionally, there was some MCT present in that sample as the MCT was not completely removed from the precipitate before analyzing it.

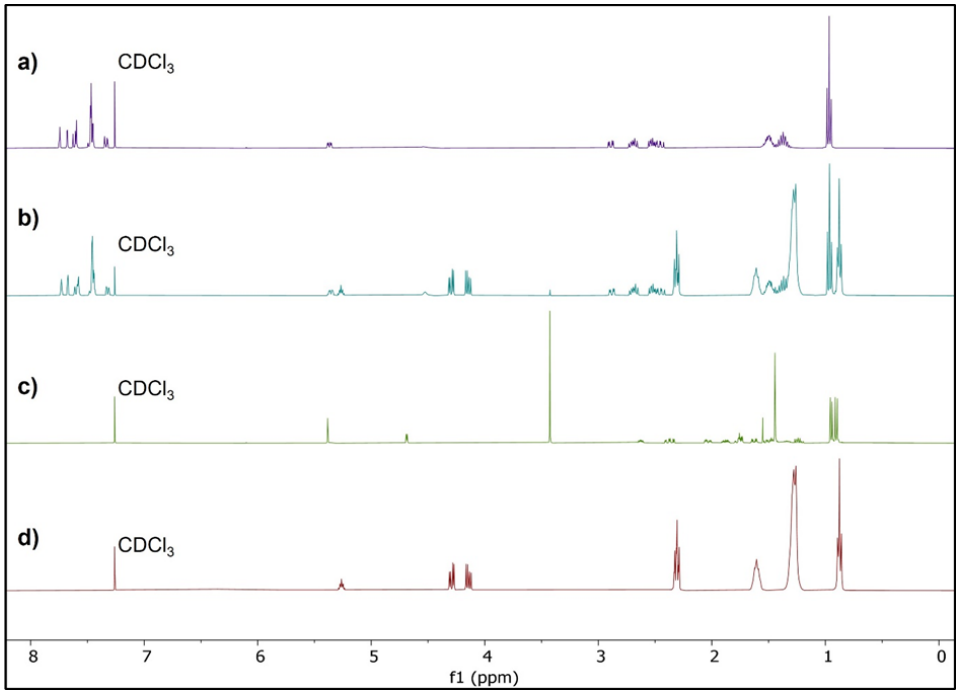

Figure 10. (a) NMR spectrum of lumefantrine on its own, (b) NMR spectrum of the precipitate that formed from a mixture of lumefantrine and artemether, (c) NMR spectrum of artemether on its own, and (d) NMR spectrum of MCT on its own. The figure was published originally in paper 1.

Another NMR comparison is shown in Figure 11 where the spectra of LF by itself (red), the precipitate of LF by itself in MCT (green), and the precipitate from the AR/LF mixture (blue) are overlayed. All spectra are calibrated to the $CDCl_3$ solvent peak at 7.26. When the spectra are compared, a small peak shift can be observed, especially in the aromatic region, for the LF peaks. This shift is small for the precipitate of LF by itself but increases when a slight amount of AR is present as well.

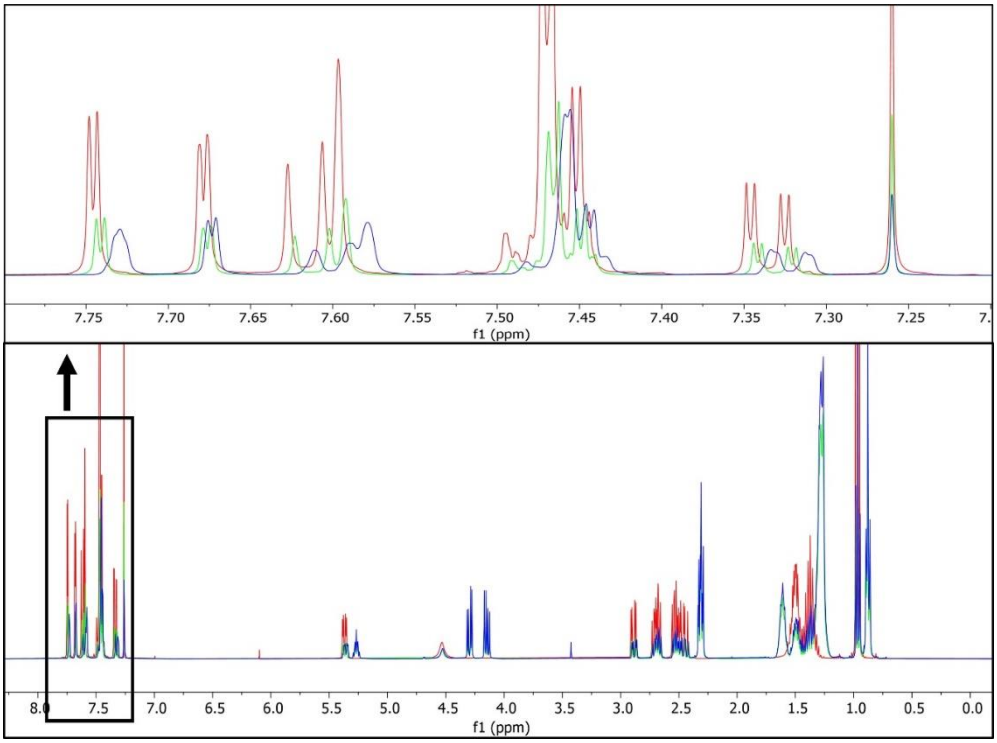

Figure 11. Overlayed NMR spectra of lumefantrine on its own (red), the precipitate formed when LF was dissolved in MCT by itself (green), and the precipitate that formed from a mixture of lumefantrine and artemether in MCT together (blue). The top shows a zoomed in version of the aromatic region, showing clearer peak shifts that were observed. All spectra were calibrated to the CDCl3 solvent peak at 7.26 ppm.

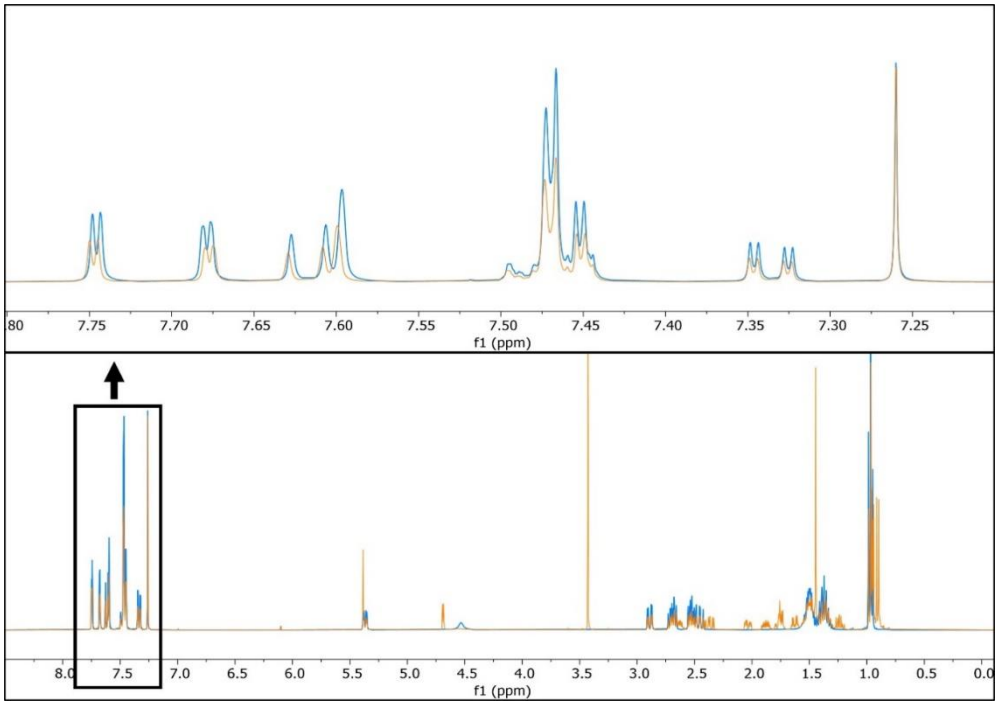

Figure 12. Overlayed NMR spectra of lumefantrine on its own (blue) and a mixture of LF and AR together in the absence of MCT (orange). The top shows a zoomed in version of the aromatic region. Both spectra were calibrated to the $CDCl_3$ solvent peak at 7.26 ppm.

When an organic acid, such as decanoic acid, was added to the formulations that were previously prepared, precipitated LF dissolved.

4.4 Formulation preparation

4.4.1 Formulation based on monounsaturated omega-9 fatty acid

Table 31 shows the results of the experiments. It was observed that LF did not dissolve in formulations that contained 4.3 to 7.7 % (0.1– 0.55 g) monounsaturated omega-9 fatty acid and were prepared at 25 °C and 43 °C after heating for more than 1 h. LF dissolved in a formulation that contained 5.56 % (0.1 g) monounsaturated omega-9 fatty acid at 60 °C but precipitation occurred in less than two weeks. Based on visual observation, all formulations that contained monounsaturated omega-9 fatty acid from 19.6 to 45.5 % (0.55 to 1.0 g) and were prepared at temperatures ranging from 25 °C to 60 °C dissolved and remained in solution for more than a year when stored at room temperature ranging from 21 to 23 °C.

Table 31. Monounsaturated omega-9 fatty acid based formulation results

Factors		Factors		Responses	
Experiment (n=3)	**Oleic acid amount (g)**	**Miglyol® 812 N**	**Temperature ±2(°C)**	**Precipitation time (months)**	**Dissolution time (min)**
F 1	0.10	1.50	25	0	N 60
F 2	1.00	2.00	43	> 12	44 ±1.53
F 3	0.55	1.50	43	>12	87 ±3.61
F 4	1.00	1.00	43	>12	45 ±5.86
F 5	0.55	1.00	25	0	N 94
F 6	0.55	1.50	43	>12	81 ±2.08
F 7	0.10	1.50	60	0.6	71 ±1.53
F 8	0.10	2.00	43	0	180 (partially dissolved)
F 9	0.55	2.00	60	>12	29 ±2.52
F 10	0.55	1.50	43	>12	75 ±2.08
F 11	0.10	1.00	43	0	N 105
F 12	0.55	2.00	25	0	150 (partially dissolved)
F 13	1.00	1.50	60	>12	18 ±3.61
F 14	1.00	1.50	25	>12	139 ±5.86
F 15	0.55	1.00	60	>12	26 ±3.51

N 60, N 94, N 105 means LF did not dissolve after heating for 60, 94 and 105 minutes respectively.

4.4.2 Statistically based design of experiment using decanoic acid

The experimental runs were designed by MODDE®13 software. Model fitting was done by multiple linear regression (MLR) to estimate coefficients of terms. The shape of the response distribution was not normally distributed and was therefore log transformed to increase the value of Q2 from -0.2 to 0.99 which represents the estimate of future prediction precision. The model was valid for both LF precipitation time and dissolution time as demonstrated by a Q2 of 0.99 and 0.93 respectively. Table 32 shows summary of fit.

Table 32. Summary of fit by MODDE® 13

	Summary of fit				Summary of fit after log transformation			
	R2	Q2	Reproducibility	Model validity	R2	Q2	Reproducibility	Model validity
LF precipitation time	0.72	-0.2	1	Missing	1	0.99	1	missing
LF dissolution time	0.8	-0.2	0.99	-0.2	0.97	0.93	0.96	0.85

Decanoic acid was the only factor that was found to be significant for the precipitation time of LF. No significant interactions were observed for the precipitation time of LF. However, the square term of decanoic acid was significant and it was observed that a decrease in decanoic acid amount shortened the precipitation time (Figure 13).

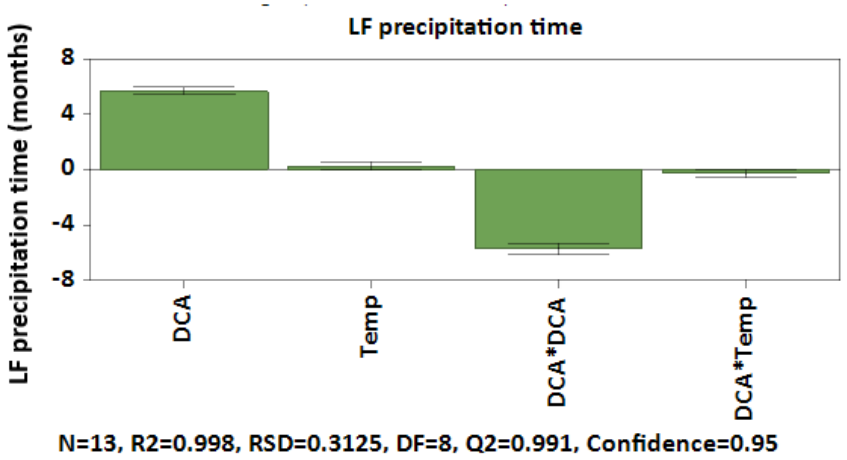

Figure 13. Coefficient plot for LF precipitation time. The figure was published in paper 1

For LF dissolution time, decanoic acid and temperature were found to be significant factors and interactions were not observed. Decreasing both the temperature and decanoic acid amount lengthens the dissolution time as shown in Figure 14. The square term of decanoic acid was found to be significant and showed that an increase in decanoic acid amount shortened the dissolution time. The regression relationship between factors and responses were significant at a *p* value <0.05.

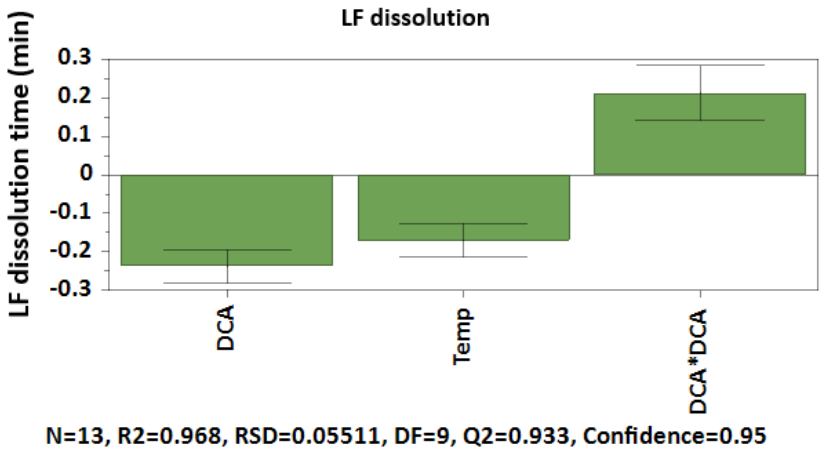

Figure 14. Coefficient plot for LF dissolution time. The figure was published in paper 1

LF did not dissolve in formulations that contained a combination of either 4.3 % DCA and 86.9% MCT or a combination of 7.7 % DCA and 76.9% MCT in the formulation after heating for more than 90 min at 35°C. However, when the same formulations were prepared at 60°C, LF dissolved in less than 60 min, but precipitation occurred shortly during storage at 21 to 23°C. For the formulation that contained 7.7% DCA and 76.9 % MCT, LF precipitated in less than two weeks while for the formulation that contained 4.3% DCA and 86.9% MCT, precipitation occurred in less than 2 months. LF dissolved in formulations that contained more than 20% DCA when prepared at 35 to 60 °C and remained in solution for more than a year. Results are summarized in Table 33.

Table 33. Proposed factor combination by MODDE® 13 and relative responses obtained (n=3)

	Factors			Responses	
Run	**Decanoic acid g (%)**	**Miglyol 812® N g (%)**	**Temperature (±°C)**	**Dissolution time (min)**	**Precipitation time (months) at 21-23°C**
1	1 (30.3)	2 (60.6)	35	36 ± 2.0	>12
2	1 (45.5)	1 (45.5)	60	13 ± 1.0	>12
3[a]	0.55 (23.9)	1.5 (65.2)	48	25 ± 1.5	>12
4[a]	0.55 (23.9)	1.5 (65.2)	48	23.7 ± 1.5	>12
5	0.1 (4.3)	2 (86.9)	60	39 ± 1.5	1.75
6	0.1 (7.7)	1 (76.9)	60	49.5 ± 2.1	0.5
7[a]	0.55 (23.9)	1.5 (62.5)	47.5	26 ± 2.0	>12
8[a]	0.55 (23.9)	1.5(65.2	48	21 ± 4.0	>12
9[b]	0.1 (7.7)	1 (76.9)	35	92	0
10[a]	0.55 (23.9)	1.5 (65.2)	48	19 ± 3.2	>12
11	1 (45.5)	1 (45.5)	35	28.7 ± 2.9	>12
12	1 (30.3)	2 (60.6)	60	16 ± 3.1	>12
13[b]	0.1 (4.3)	2 (86.9)	35	97	0

[a] Center points, [b] Lumefantrine did not dissolve within the specified period

4.4.2.1 Optimization of the decanoic acid based formulation

The response contour plot in Figure 15 shows that decreasing decanoic acid amount shortens the precipitation time.

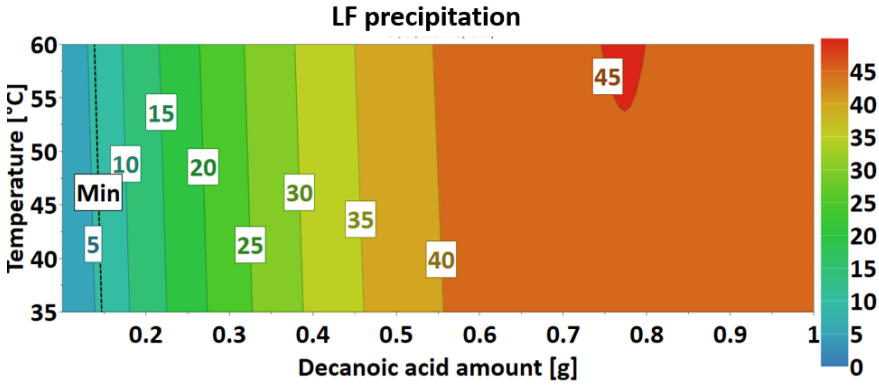

Figure 15. Response contour plot for LF precipitation time. The figure was published in paper 1.

The response contour plot in Figure 16 shows that decreasing decanoic acid amount slows the dissolution process.

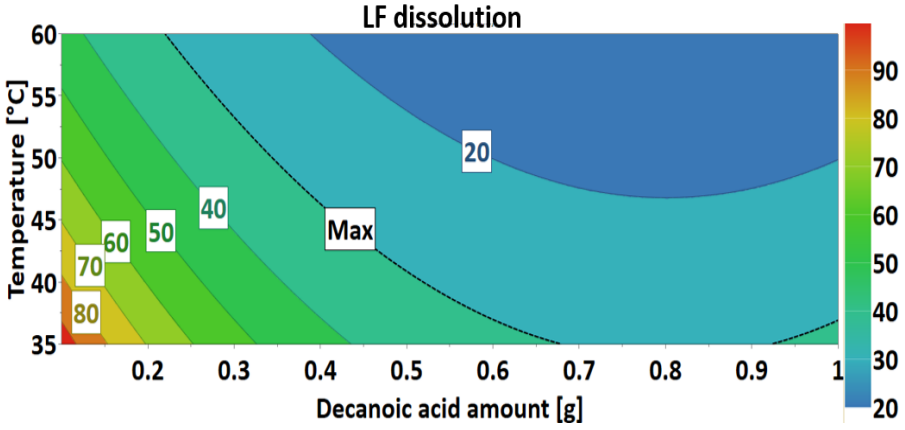

Figure 16. Response contour plot for LF dissolution time. The figure was published in paper 1

Optimization using the sweet spot plot showed that to maximize the time that LF should remain solubilized at least more than 10 % decanoic acid must be included in the formulation regardless of the temperature (35 °C to 60 °C) at which the formulations are prepared as shown in Figure 17 given that there were no prediction errors by the model.

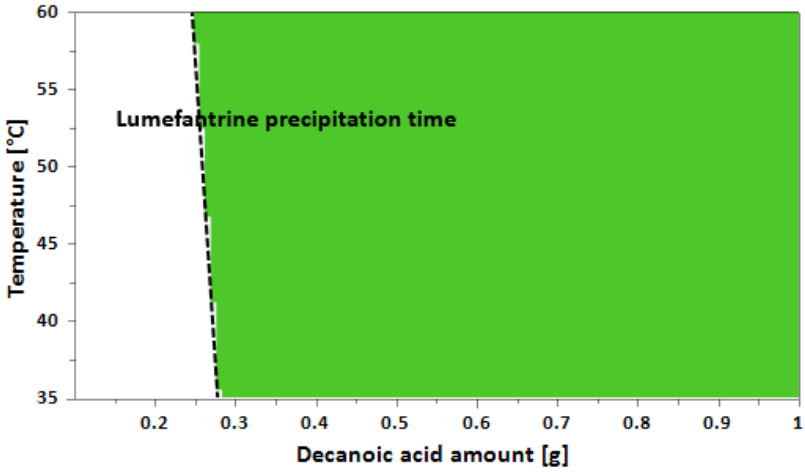

Figure 17. Sweet spot for LF precipitation time showing all possible areas (green) where the formulation can be optimized.

The design space plot also showed that the probability of LF precipitating in the formulation is greater than 50% when less than 10% (less than 0.3g) decanoic acid is used in the formulation as shown in Figure 18. The default acceptance level in MODDE® is 1% prediction fail rate and was used but can also be changed to suit the desired objective.

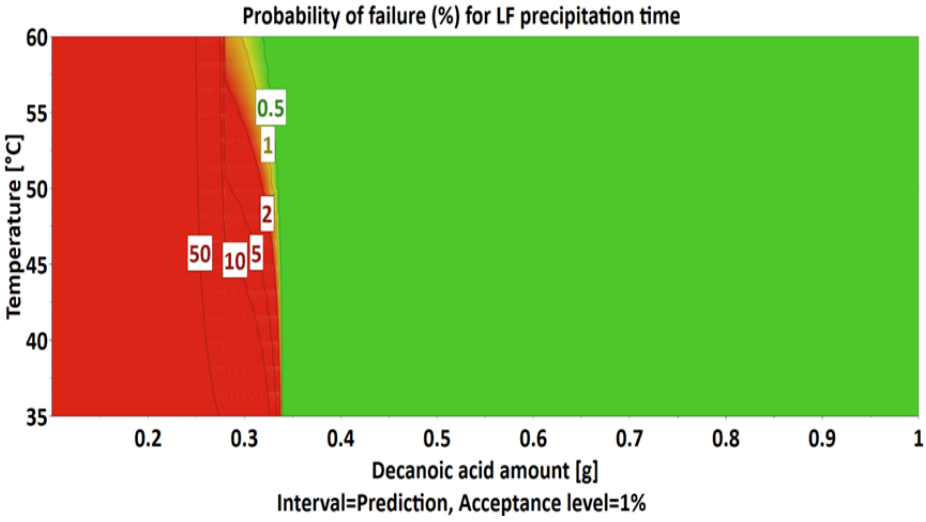

Figure 18. Design space plot showing the projected sweet spot area with a defined probaility to fail included

The desirability for LF precipitation time also showed that long precipitation time was achieved when the amount of decanoic acid was at least 0.3 g or (greater than 10 %) (Figure 19).

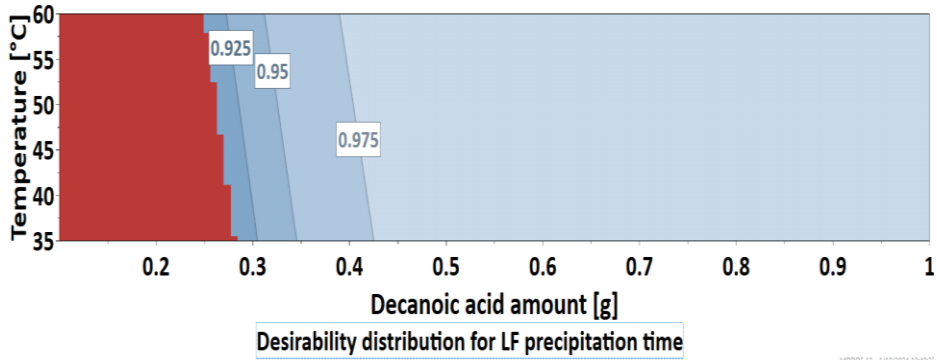

Figure 19. Desirability plot showing where objective targets are met

Visualizing the output using the factor effect plot (Figure 20) showed that increasing the amount of decanoic acid made LF remain solubilized for a longer period and the dissolution time was shortened.

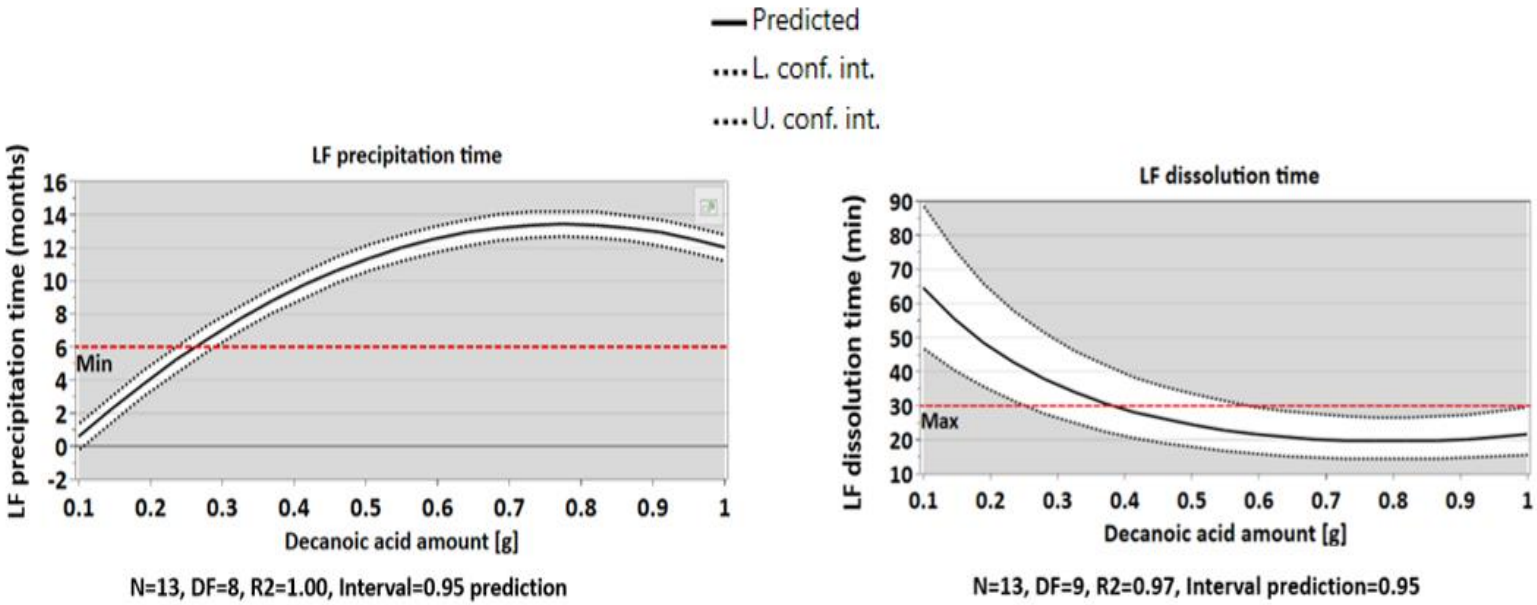

Figure 20. Factor effect plot showing how the predicted values of the selected response vary when the factor changes over its range, all other factors in the design held constant at their averages

The overlay prediction plot showed that an increase in decanoic acid amount lengthened the precipitation time and reduced the dissolution time. An increase in temperature decreased the dissolution time but had no effect on the precipitation time. MCT had no influence on both the precipitation time and the dissolution time (Figure 21).

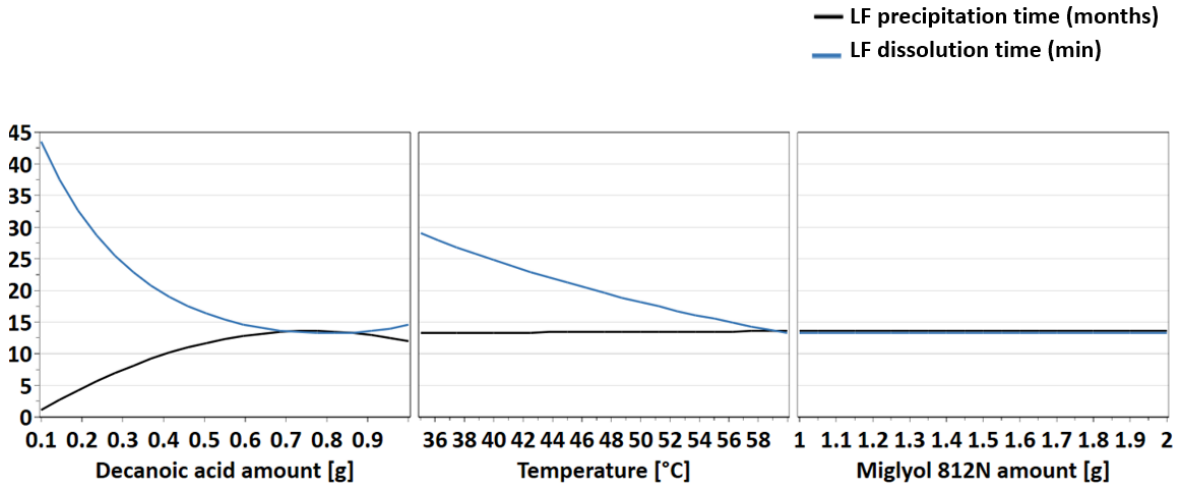

Figure 21. The overlay prediction plot showing the predicted values for the responses without displaying confidence intervals

4.4.3 Formulation containing 10 % of a mixture of decanoic and monounsaturated omega-9 fatty acids

These experiments were not designed and analyzed using MODDE®. It was observed visually that LF dissolved in all the formulations and remained in solution for more than a year. Table 34 shows the results of experiments.

Table 34. Formulation containing 10 % of a mixture of decanoic acid and monounsaturated omega-9 fatty acid in different ratios

	Factors					Responses	
Formulation (n=3)	**Decanoic acid (%)**	**Oleic acid (%)**	**Miglyol® 812 N (%)**	**Temperature ±2 °C**	**Kolliphor® PS 20 (%)**	**Precipitation time (months)**	**Dissolution time (min)**
1	7.5	2.5	89.8	60	0.2	> 12	23 ±2.00
2	7.5	2.5	89.5	60	0.5	> 12	25 ±1.00
3	5	5	89.8	60	0.2	> 12	28 ±2.08
4	5	5	89.5	60	0.5	> 12	36 ±6.35
5	2.5	7.5	89.8	60	0.2	> 12	33 ±3.89
6	2.5	7.5	89.5	60	0.5	> 12	34 ±4.51

4.4.4 Formulation containing a mixture of decanoic and monounsaturated omega-9 fatty acids (1 %, 5%, 10% and 15 %) in a 1:1 ratio

It is important to note that these experiments were not designed and analyzed using MODDE®. The results in Table 35 show that LF dissolved in all samples containing a mixture of decanoic acid and oleic acid. However, precipitation occurred within 2 weeks for samples that contained a total of 1% of the organic acids. For samples that contained 5% of the organic acids, LF was partially precipitated within 2 weeks and completely precipitated within 5 months. LF did not precipitate in formulations that contained 10% and 15% of the organic acids. These formulations did not contain mPEG 350.

Table 35. Formulation containing a mixture of decanoic acid and monounsaturated omega-9 fatty acid in a 1:1 ratio

	Factors					Responses	
Formulation (n=3)	**Decanoic acid (%)**	**Oleic acid (%)**	**Miglyol® 812 N (%)**	**Temperature ±2 °C**	**Kolliphor® PS 20 (%)**	**Precipitation time (months)**	**Dissolution time (min)**
F 1	0.5	0.5	92	60	0.2	0.5	78 ±2.65
F 2	0.5	0.5	92	60	0.5	0.5	76 ±1
F 3	2.5	2.5	89	60	0.2	0.5 (partial) & at 5 months complete precipitation	77 ±3.51
F 4	2.5	2.5	88	60	0.5	0.5 (partial) & at 5 months complete precipitation	90 ± 16.97*
F 5	5	5	84	60	0.2	40	61 ±1.53
F 6	5	5	84	60	0.5	40	56 ±4.01
F 7	7.5	7.5	79	60	0.2	41	23 ±1.15
F 8	7.5	7.5	79	60	0.5	41	25 ±3

*n=2

4.4.5 Formulation containing varying quantities of mPEG 350, decanoic acid and Miglyol® 812 N

LF did not dissolve in formulations that contained greater than 5% of mPEG 350 at 35°C. LF also did not dissolve in formulations that contained DCA to mPEG ratio of: 5 to 32%, 0.99 to 87% and 19 to 71% at 60°C. LF partially dissolved in formulations that contained 11% and 43% DCA and 43% mPEG 350 at 35°C. LF dissolved in a formulation that contained 53% DCA and 14% mPEG 350 at 60°C within 15 minutes. Results can be seen in Table 36.

Table 36. Formulations containing varying amounts of mPEG 350, 350, decanoic acid and Miglyol® 812 N

Formulation (n=3)	Factors				Responses	
	Decanoic acid (%)	**Miglyol® 812 N (g)**	**mPEG 350**	**Temperature ±2 °C**	**Precipitation (months)**	**Dissolution time (min)**
F 1	5	31	31	35	0	N (50)
F 2	1	6	87	35	0	N (54)
F 3	14	55	14	35	0	N (54)
F 4	19	5	71	35	0	N (60)
F 5	1	87	6	35	0	N (60)
F 6	0.6	48	48	35	0	N (57)
F 7	19	71	5	35	0	N (56)
F 8	11	43	43	35	0	N (50 very partially dissolved)
F 9	5	32	32	60	0	N (45)
F 10	0.99	6	87	60		
F 11	53	15	14	60	Dissolved	15 ±2.08

N = did not dissolve after heating for at least 50 min

4.4.6 Quantification of artemether and lumefantrine in lipid vehicles

4.4.6.1 Determination of lambda max

The results of the 5-fold diluted LF solution when scanned from 275 to 325 nm showed maximum absorbance at 302 nm and when scanned from 190 to 400 nm showed maximum absorbance at 234 nm. The stock solution of LF when scanned from 275 to 325 nm showed similar absorbance at 292, 298, and 307 nm. The stock solutions when scanned from 190 to 400 nm showed similar absorbance at 258, 276, and 314 nm for LF. The 5-fold diluted solution of AR when scanned from 190 to 400 nm showed maximum absorbance at 206 nm. The stock solutions, when scanned from 190 to 400 nm showed maximum absorbance at 218 nm for AR. The stock solutions showed higher absorbance readings than the diluted solutions for both APIs.

4.4.6.2 HPLC quantification method

Isocratic elution using the Phenomenex® Luna® 5 µm 100 Å 250 x 4.6 mm and 0.1% TFA in methanol and 0.1% TFA in water as the mobile phase

Wavelength was the only significant factor for separating AR from excipients and no interaction was observed as shown in Figure 22. Some nonsignificant terms were therefore excluded to improve the model. MeOH and flow rate were the significant factors and the interaction between MeOH and wavelength was the only significant interaction observed. The square term of MeOH was significant (Figure 22). Both models for AR and LF separation were not good.

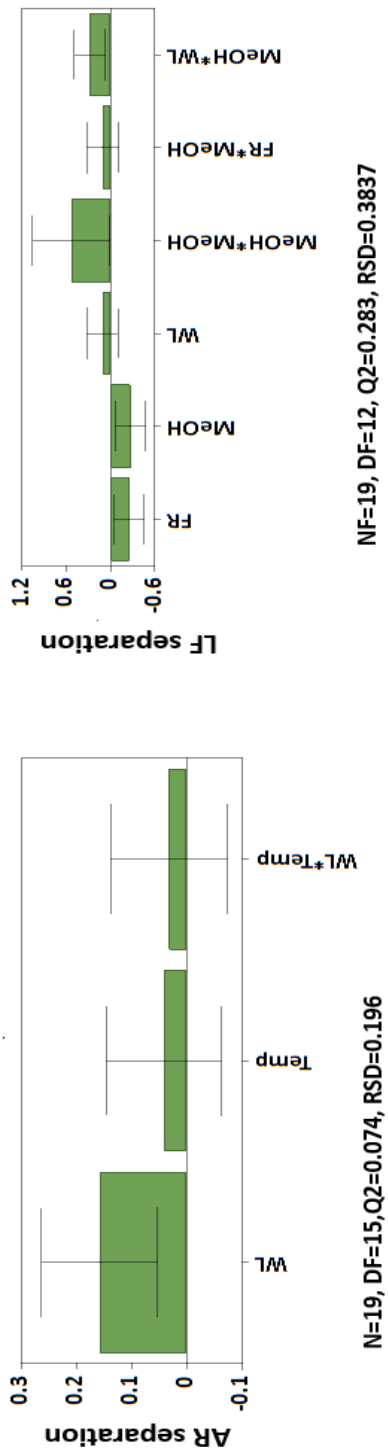

Figure 22. Coefficient plots showing significant and nonsignificant factors and interactions and a square term for AR and LF separation from excipients

Comparing Luna® 5µm C18(2) 100 Å 250 x 4.6 mm and the Kinetex® 5 µm C8 100 Å 150 x 4.6 mm Phenomenex columns in separating the AR and LF from the excipients

Following results of initial experiments, the next step was to compare the two columns to select a better column for the analysis. The columns were used to find a column that retains APIs well, as well as to achieve good separation between the APIs and excipients. The histograms were autotransformed (log transformed) and non-significant factors were excluded. Both columns were not very good at separating the APIs from the excipients using 0.1 % formic acid in ACN and 0.1 % formic acid in water. The significant factors for AR retention were the mobile phase compostion, column, flow rate and temperature. An increase in ACN, FR decreased the retention time to differing extent depending on the type of column that was used. The significant interaction for AR retention was only between ACN and column. For separating AR from excipients the significant factors were column, flow rate and ACN. The interaction between the flow rate and temperature was found to be significant. Results are shown in Figure 23. The models for AR retention and separation were both significant and good.

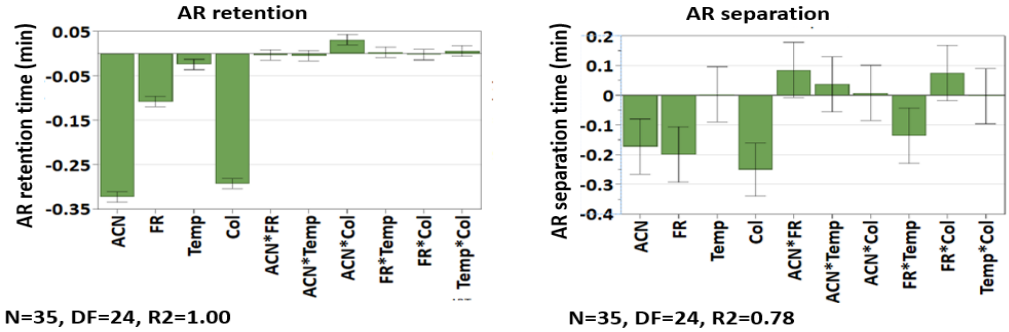

Figure 23. Coefficient plots showing significant and nonsignificant factors and interactions for artemether retention and separation using the Lunar C18(2) and Kinetex columns

For LF retention time the significant factors were ACN, flow rate, and column and the significant interaction was only between ACN and the column. For LF separation, the significant factors were ACN, column, and flow rate. No significant interactions were observed (Figure 24).

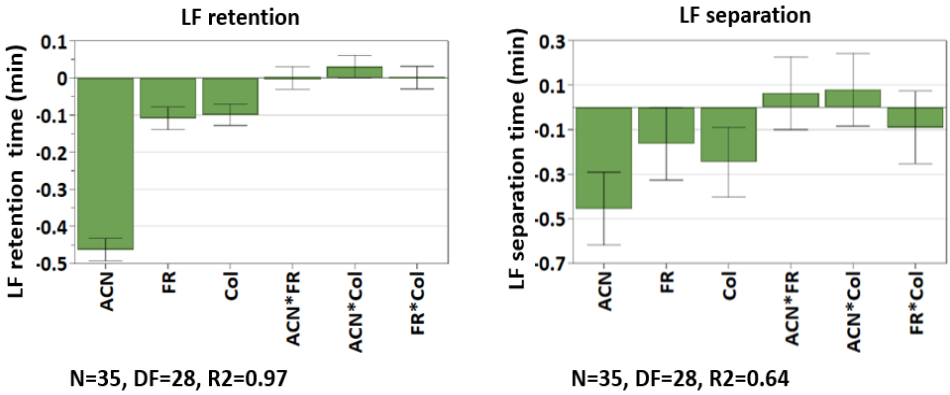

Figure 24. Coefficient plots showing significant and nonsignificant factors and interactions for lumefantrine retention and separation using the Lunar C18(2) and Kinetex columns

Gradient elution using the Kinetex column and 0.1 % formic acid in both acetonitrile and water

None of the four gradient elution methods was successful in separating the APIs from the excipients at all three wavelengths (218, 236 and 254 nm) at 25 ±2 °C. Both APIs eluted very early (1.60 to 3.76 min retention time) and merged with solvent peaks.

Isocratic elution using the *Phenomenex Luna® 5 µm C18(2) 100 Å 250 x 4.6 mm and 0.1% TFA in both acetonitrile and water*

Following the gradient elution results, an isocratic method using the Luna C18(2) was designed. A total of 19 experiments were designed using MODDE® 13 and conducted to find a method of separating APIs from the excipients (oleic acid, decanoic acid, miglyol®812 N, Kolliphor® PS 20 and mPEG 350). The shape of the response distribution (histogram) for AR seperation was autotransformed and some nonsignificant factors were excluded. Wavelength was the only significant factor for AR seperation from excipients. Increasing the wavelength resulted in increased seperation. The interactions between ACN and wavelength and between ACN and temperature were the only significant interactions observed as seen in Figure 25.

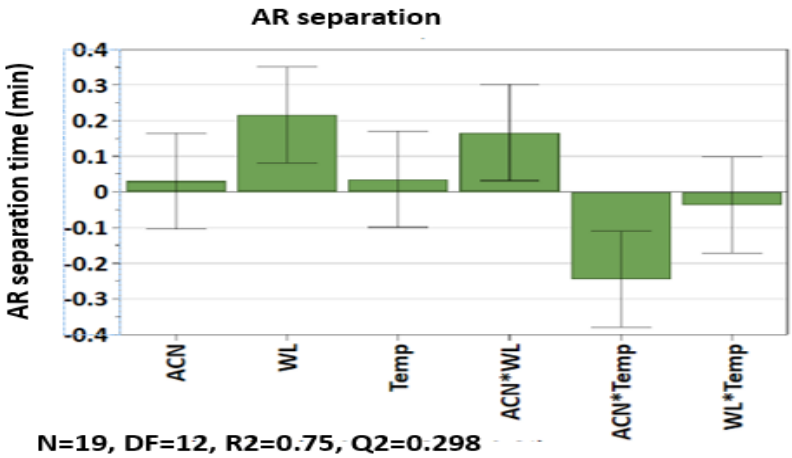

Figure 25. Cofficient plot showing significant and non significant factors and interactions for artemether separation using 0.1 % TFA in ACN and the Luna column

The model for AR separation from excipients was significant but not good as seen by a low value of Q2 in Figure 26. The model validity was also missing (Figure 27) and the variability of the replicates was very small as seen in Figure 28.

Figure 26. Summary of fit plot showing the quality of the model for AR separation from excipients using 0.1 % TFA in ACN and the Lunar C18(2) column

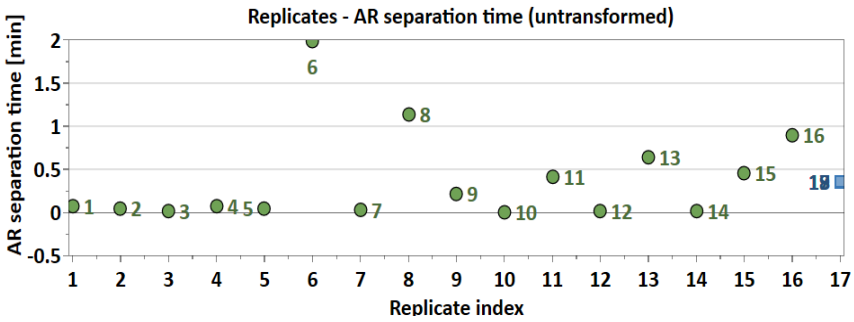

Figure 27. Replicate plot showing variation in results for all experiments. Repeated experiments are shown in a different colour (blue) connected by a line

The model for LF seperation from excipients was both significant and good and therefore, the histogram was not transformed but non-significant factors of the coefficient plots were excluded. The % of organic phase (ACN) and the flow rate were significant and the only significant interaction was between ACN and FR as shown in Figure 28. Decreasing both ACN and the FR increased separation time between LF and excipients.

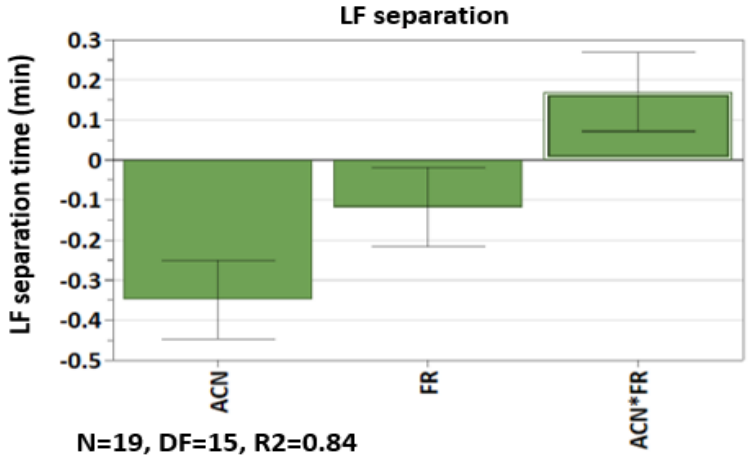

Figure 28. Coefficient plot showing significant factors and an interaction for lumefantrine separation from excipients using 0.1 % TFA in ACN and the Lunar C18(2) column

Results also showed that the model was reproducible and model validity could not be calculated as seen in Figure 29. Figure 30 shows the variability of the replicates was very small.

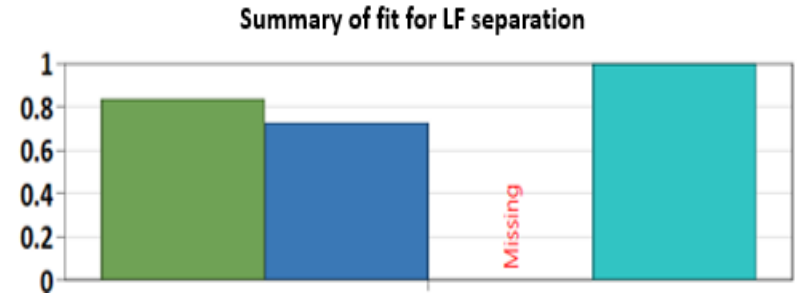

Figure 29. Summary of fit plot showing the quality of the model for LF separation from excipients using 0.1 % TFA in ACN and the Lunar C18(2) column

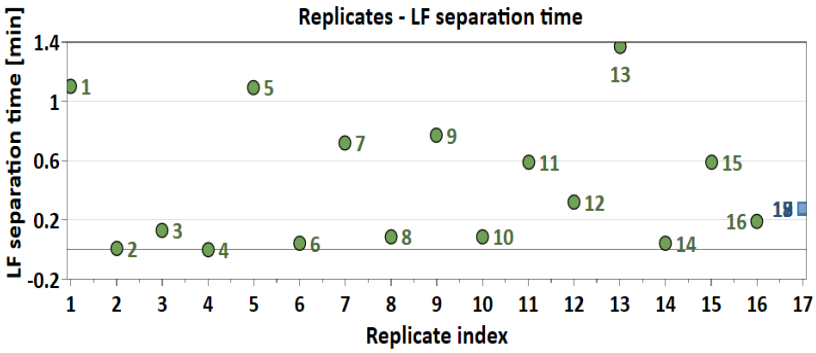

Figure 30. Replicate plot showing the variation in results for all experiments

For separating AR and LF, results showed that the histogram plot assumed a normal distribution and therefore did not require further transformation. The model was significant and good for seperating AR and LF. ACN and flow rate were the only significant factors observed and the interaction between ACN and flow rate was the only significant interaction observed as shown in Figure 31. Two square terms were detected (ACN*ACN and FR*FR). When either one of them was included in the model, Q2 increased from 0.81 to 0.97. Therefore, only the square term of ACN*ACN was included. Increasing the percentage of the organic phase and decreasing the flow rate increased the separation time between AR and LF with LF always eluting first.

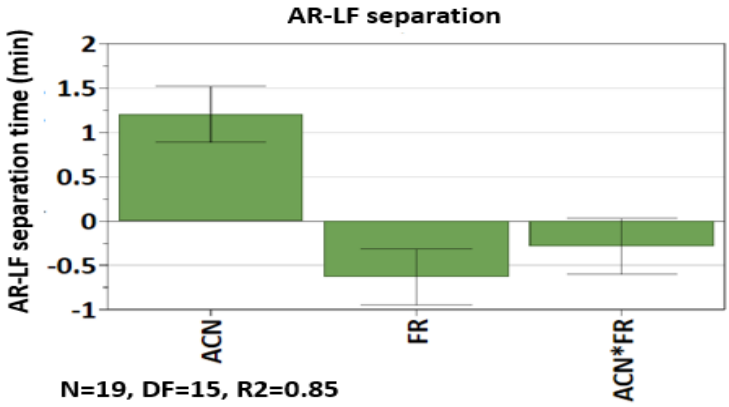

Figure 31. Coefficient plot showing significant factors and a nonsignificant interaction for AR-LF separation using 0.1% TFA in ACN and the Luna® C18(2) column

Generally, monounsaturated omega-9 fatty acid showed many peaks and interference at wavelengths below 336 nm and therefore, the same results were reanalyzed by excluding monounsaturated omega-9 fatty acid as an excipient.

Reanalysis of results of AR and LF separation from excipients using data from the isocratic elution method using the Luna® C18(2) column

Decanoic acid, MCT, polysorbate 20, mPEG 350 were the excipients being seperated from AR and LF during this reanalysis. The histogram for AR separation did not assume a normal distribution and was therefore autotransformed. The model was significant. To separate AR from excipients, wavelength was the only significant factor and lack of interaction was observed (Figure 32).

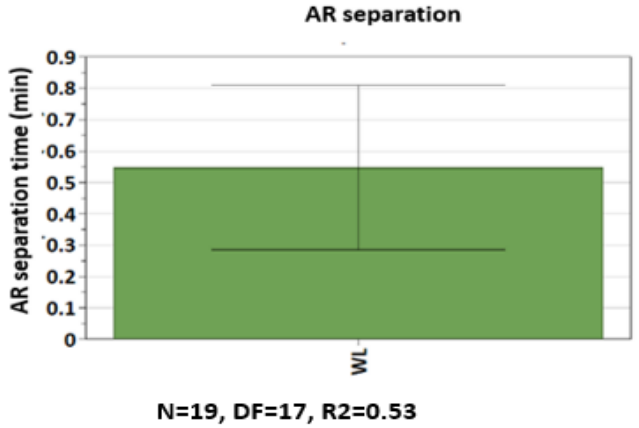

Figure 32. Coefficient plot showing a significant factor for AR separation after excluding oleic acid as an excipient in the analysis

For lumefantrine separation from excipients, the model was autotransformed to assume a normal distribution. ACN and wavelength were the only significant factors and the interaction between ACN and wavelength was significant. Decreasing the organic content and increasing the wavelength increased seperation from excipients as shown in Figure 33.

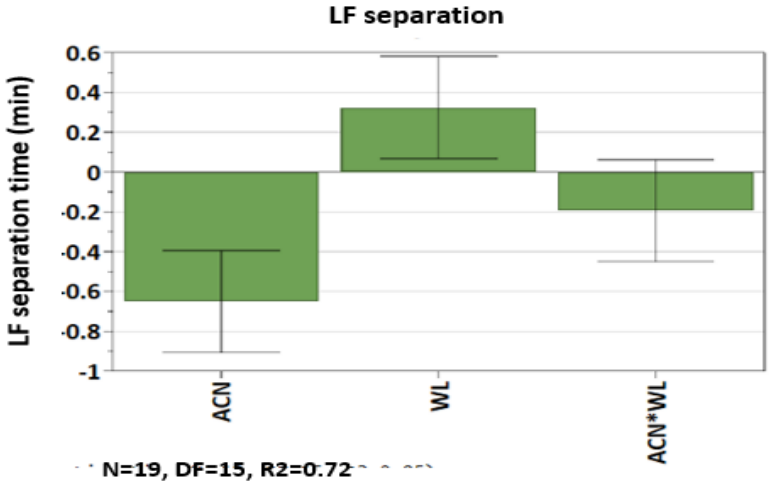

Figure 33. Coefficient plot showing significant factors for LF separation from excipients after excluding monounsaturated omega-9 fatty acid as an excipient

Optimization of HPLC method using the optimizer tool of MODDE

Based on results that were analyzed after excluding monounsaturated omega-9 fatty acid, the optimizer recommended setpoints based on the conditions and objectives of responses as well as the roles of factors that were specified. Using 65% acetonitrile at 336 nm and at 25°C was one of the setpoint that was recommended (Tables 37 and 38).

Table 37. Factors and roles that were specified and the experimental conditions recommended by optimizer

Factor	Role	Low limit	High limit	Recommended value to use	Factor contribution
ACN (%)	Free	65	90	65	53
Temperature (°C)	Free	25	40	25	
WL (nm)	Free	218	336	336	47
FR (mL/min)	Free	0.8	1.3	0.8	0

Table 38. Conditions and objectives for the responses and the predictions when the roles of all factors were specified to be free

Response	Condition	Objective	Minimum	Predicted maximum (min)	Probability of failure (%)
LF retention (min)	Observed	Predicted		20	
AR retention time (min)	Observed	Predicted		22	
LF separation time (min)	Required	Maximize	2	6	20
AR separation time (min)	Required	Maximize	1	3	22
AR-LF separation time (min)	Observed	Predicted		5	

When ACN % was specified to be constant it was recommended to use 78 % ACN as an organic modifier. Recommendations and predictions are shown in Tables 39 and 40.

Table 39. Factors and roles that were specified and the experimental conditions recommended by optimizer

Factor	Role	Low limit	High limit	Recommended value to use	Factor contribution
ACN (%)	Constant			78	0
Temperature (°C)	Free	25	40	28	
WL (nm)	Free	218	336	336	100
FR (mL/min)	Free	0.8	1.3	0.995	0

Table 40. Conditions and objectives for the responses and the predictions made when ACN % was specified to be constant

Response	Condition	Objective	Minimum	Predicted maximum (min)	Probability of failure (%)
LF retention (min)	Observed	Predicted		11	
AR retention time (min)	Observed	Predicted		15	
LF separation time (min)	Required	Maximize	2	1	
AR separation time (min)	Required	Maximize	1	3	21
AR-LF separation time (min)	Observed	Predicted		3	

When both ACN and FR were specified to be constant, it was also recommended to use 78 % ACN. Results are shown in Tables 41 and 42.

Table 41. Factors and roles that were specified and recommendations made by optimizer

Factor	Role	Low limit	High limit	Recommended value to use	Factor contribution
ACN (%)	Constant			78	0
Temperature (°C)	Free	25	40	30	
WL (nm)	Free	218	336	336	100
FR (mL/min)	Constant			1.1	0

Table 42. Conditions and objectives for the responses and the predictions made when ACN % and FR were specified to be constant

Response	Condition	Objective	Minimum	Predicted maximum (min)	Probability of failure (%)
LF retention (min)	Observed	Predicted		9	
AR retention time (min)	Observed	Predicted		12	
LF separation time (min)	Required	Maximize	2	1	
AR separation time (min)	Required	Maximize	1	3	22
AR-LF separation time (min)	Observed	Predicted		3	

From previous results of DOE, it was already observed that separation was possible under these conditions, but the limitation of using 65 % ACN was the very poor peaks detected and the long run times. However, analysis was still done using alternative flow rates (0.9, 1.1, and 1.2 mL/min) at 40°C and at both 218 and 336 nm just to compare results. These results also showed that AR separation was possible using 65% ACN at a FR of 1.1 mL/min at 218 nm but very poor peaks were detected, and long retention time of 15 min. From several previous runs AR peak area is greatly diminished as it is detected as noise at 336 nm as seen in (Figure 34). At 218 nm the peak is better though not very good but quantification at this wavelength is possible as seen in Figure 35.

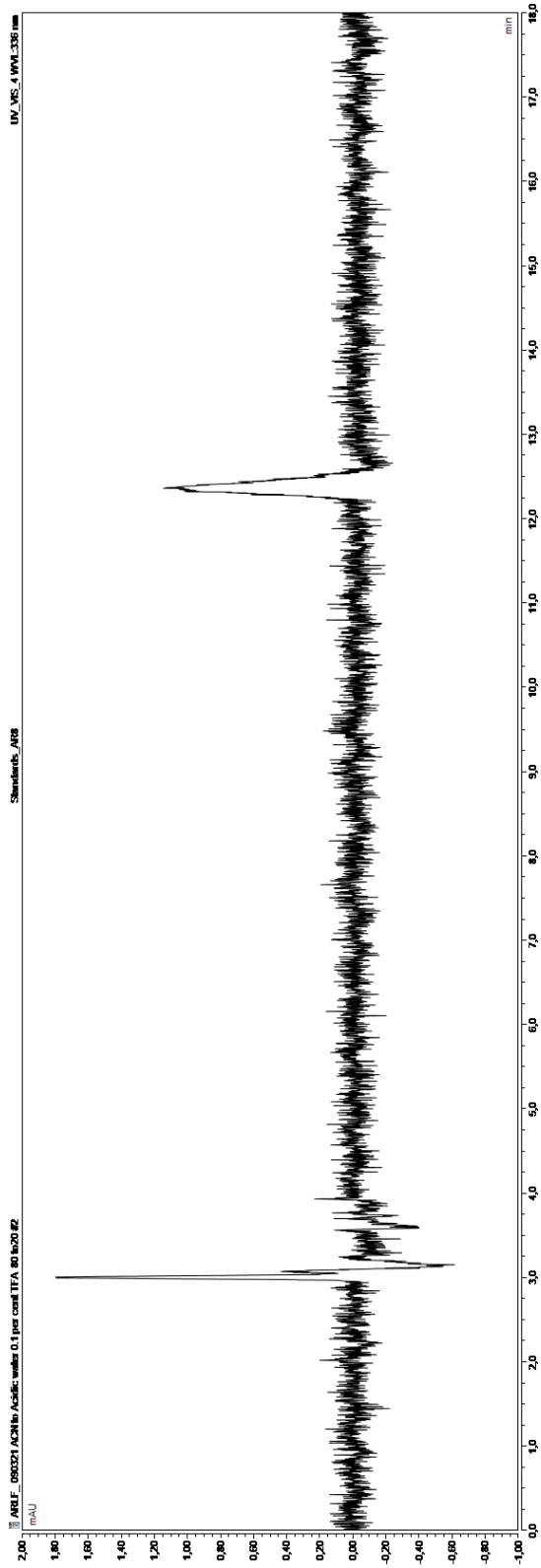

Figure 34. Chromatogram of artemether standard when analyzed at 336 nm

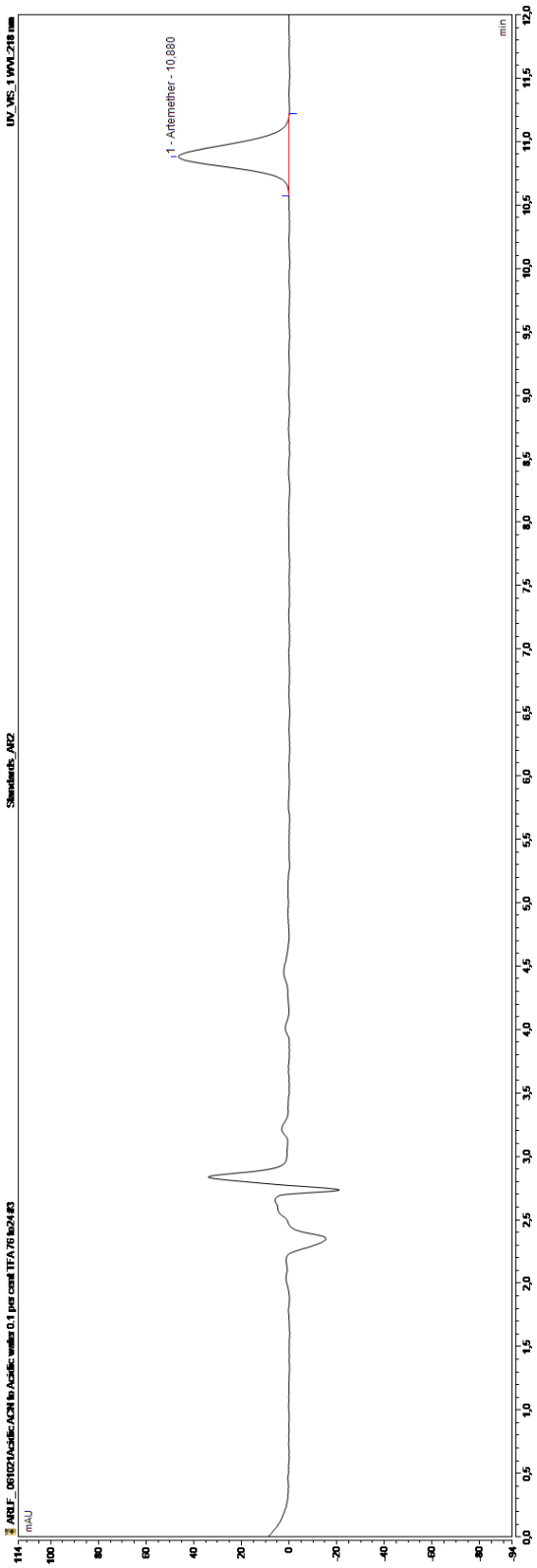

Figure 35. Chromatogram of artemether standard when analyzed at 218 nm

For LF separation, it was possible at 0.9 and 1.1 mL/min at both 218 and 336 nm with highest separation achieved at a slow FR of 0.9 mL/min at 336 nm (Figure 36). The other recommended setpoints were to do the analysis using 78% ACN at 336 nm. These runs were not conducted because at 336 nm AR is almost not detected and 78% is very close to 80% ACN where separation failed from previous experiments.

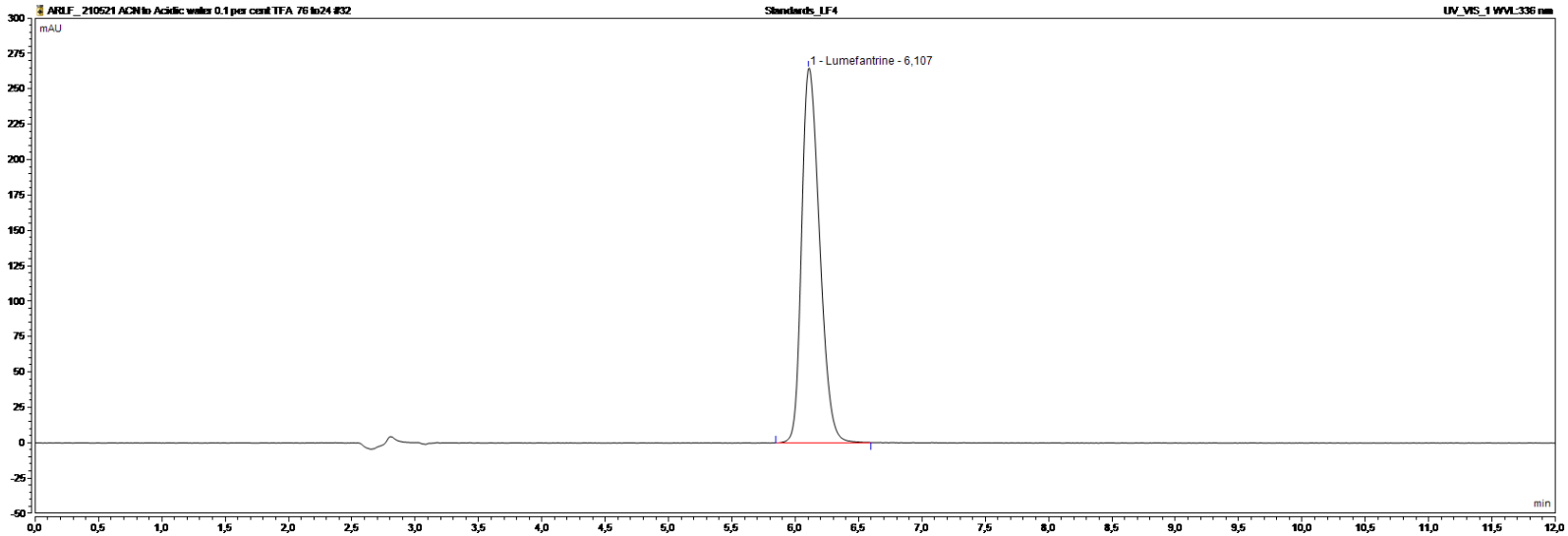

Figure 36. Chromatogram of lumefantrine standard when analyzed at 336 nm

Therefore, the contour plot was utilized to find the optimal point. It was observed that good separation for artemether could be achieved above 300 nm regardless of the FR (0.8, 1.05, and 1.3 mL/min) and organic modifier content (65 to 90%) used. Below 290 nm, very little separation (less than 1 min) was observed Figure 37.

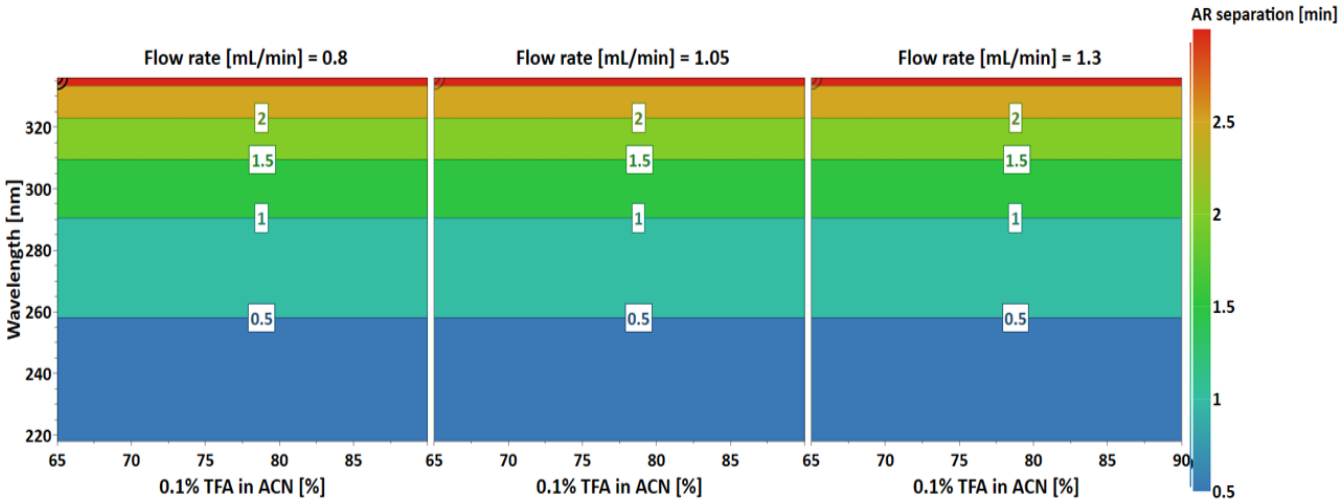

Figure 37. Contour plot showing artemether separation from excipients after excluding momounsaturated omega-9 fatty acid

For LF separation, it was observed that good separation was possible when less than 70% ACN was used, and highest separation was at 336 nm (Figure 38).

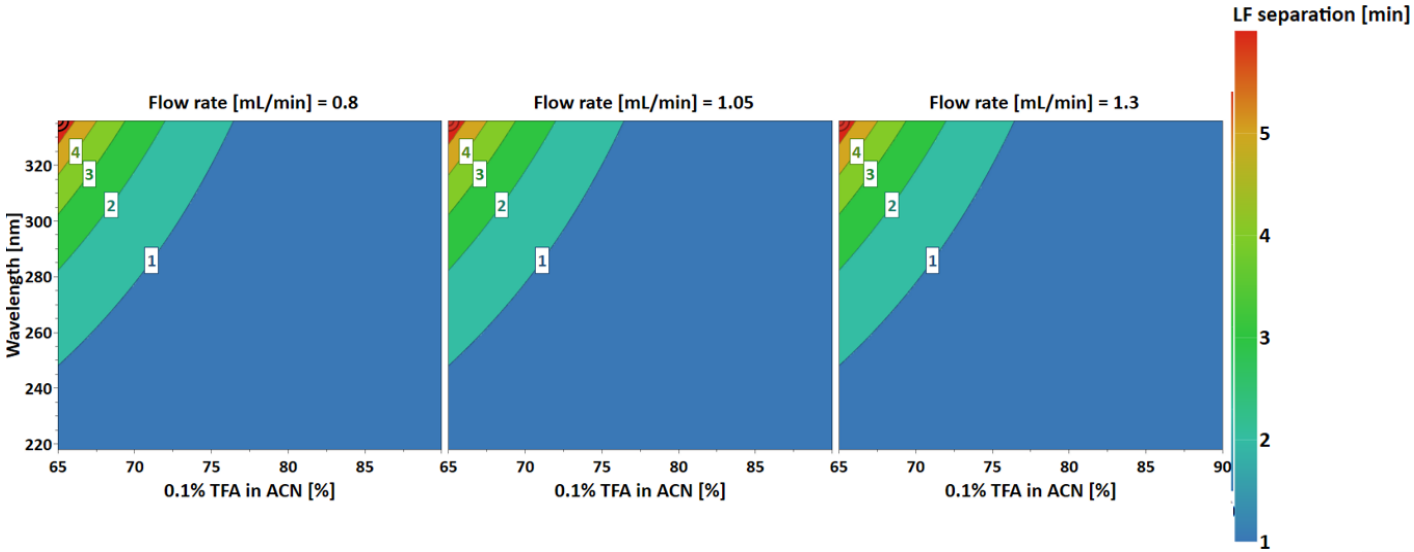

Figure 38. Contour plot showing lumefantrine separation from excipients when monounsaturated omega-9 fatty acid was excluded

For both AR and LF separation from excipients, the contour plot showed same set point (65% ACN, 0.8 mL/min, 336 nm at 40°C). However, considering the limitations of analyzing AR and LF under such conditions, it was decided to try to run samples using 76% acetonitrile. Therefore, selected runs were conducted, and results are shown in Tables 43 and 44 for artemether and lumefantrine respectively.

Table 43. Artemether separation from excipients

0.1% TFA in ACN	FR	WL (nm)	Temperature °C	LF RT	AR RT	SEP AR DCA	SEP AR MIG	SEP AR KPS 20	SEP AR mPEG	SEP AR LF
76	0.8	218	25	12.13	18.79	0.04	0.78	1.16	0.34	6.66
76	0.8	336	25	12.14	18.77	16.01	15.58	15.09	15.09	6.63
76	0.8	218	40	8.03	13.78	0.81	3.99	1.33	0.84	5.75
76	0.8	336	40	8.03	13.75	4.54	3.96	10.37	10.05	5.72
76	**1.1**	**218**	**25**	**7.70**	**12.12**	**1.21**	**2.63**	**1.54**	**2.58**	**7.70**
76	1.1	336	25	7.70	12.08	4.20	9.55	1.50	9.14	4.38
76	1.1	218	40	5.56	9.68	0.11	0.15	0.14	0.47	4.12
76	1.1	336	40	5.56	9.65	3.18	7.24	7.23	7.64	4.09
76	1.2	218	25	6.20	9.57	1.09	1.80	0.37	2.51	3.37
76	1.2	336	25	6.20	9.55	3.09	7.47	7.46	5.98	3.35
76	1.2	218	40	4.55	7.98	0.51	0.04			3.43
76	1.2	336	40	4.55	7.96	5.96	5.95			3.41

SEP means separation, KPS 20 means kolliphor® PS20 (polysorbate 20)

Table 44. Lumefantrine separation from excipients

0.1% TFA in ACN	FR	WL (nm)	Temperature °C	LF RT	AR RT	SEP AR DCA	SEP AR MIG	SEP AR KPS 20	SEP AR mPEG	SEP AR LF
76	0.8	218	25	12.13	18.79	0.21	0.95	1.16	1.62	6.66
76	**0.8**	**336**	**25**	**12.14**	**18.77**	**9.38**	**8.95**	**8.46**	**8.46**	**6.63**
76	0.8	218	40	8.03	13.78	1.12	1.76	0.45	0.47	5.75
76	0.8	336	40	8.03	13.75	1.18	4.65	4.65	4.33	5.72
76	**1.1**	**218**	**25**	**7.70**	**12.12**	**0.24**	**1.05**	**0.95**	**0.81**	**4.42**
76	1.1	336	25	7.70	12.08	0.18	5.17	5.17	4.76	4.38
76	1.1	218	40	5.56	9.68	0.08	0.48	0.19	0.56	4.12
76	1.1	336	40	5.56	9.65	0.91	3.15	3.14	3.55	4.09
76	1.2	218	25	6.20	9.57	0.3	0.46	0.7	0.7	3.37
76	1.2	336	25	6.20	9.55	0.26	4.12	4.11	2.63	3.35
76	1.2	218	40	4.55	7.98	0.42	0.09			3.43
76	1.2	336	40	4.55	7.96	2.55	2.54			3.41

Finally, the chosen optimized method for artemether was acetonitrile: water (0.1% TFA in whole mobile phase) 76:24%, 1.1 mL/min at 218 nm and 25°C. The same mobile phase composition was able to separate lumefantrine from excipients but using a flow rate of 0.8 mL/min at 336 nm at both temperatures of 25°C and 40°C. Highest separation for LF was at 25°C, however due to long retention time (12 min) and thus long run time, LF was chosen to be analyzed at 40°C (retention time 8 min). It was possible to quantify AR from prepared formulations using the optimized method. LF failed to be quantified accurately as only less than 20% of the stated content could be reproductively quantified. However, it was possible to quantify LF at 336 nm using the UV spectrophotometer and using the Dionex Ultimate 3000 HPLC, Ultimate 3000 LPG-3400A pump, Ultimate 3000 autosampler, Ultimate 3000 Variable Wavelength Detector (VWD)- 3400 at ambient temperature and mobile phase 0.1% TFA in ACN and 0.1% TFA in water (76/24% V/V), 0.8 mL/min, 20µL injection volume at 336 nm. The pH of the water phase was 2.2 ±1 at 24 °C.

The linearity for AR was determined at eight concentration levels, that ranged from 100 to 500 µg/mL. At concentrations below 100 µg/mL, too much noise was observed such that it was difficult to differentiate between AR peak and noise. The regression coefficient (R^2 = 0.9993) showed that the peak areas were directly proportional to AR concentration in the concentration range that was evaluated and therefore, the optimized method showed acceptable linearity. For LF, linear correlation between concentrations and peak areas was observed in the range 0.2 to 25 µg/mL (R2 = 0.9997.

4.5 Bioequivalence study of the lumefantrine rectal enema and the commercially available generic oral suspension. A pilot study.

Six healthy NZW rabbits (two male and four female non pregnant) weighing between 3.75 and 5.14 kg participated in the study. Generally, both the reference and test formulations seemed to be well tolerated in the animals, as no signs indicated discomfort or irritation. It was not easy to withdraw 1.2 mL of blood at certain time points. It was also observed that under fasted conditions animals took (swallowed) the oral formulations better than under fed state. Rabbit B spit out some formulation i.e. some formulation came out of the mouth) after administration under fed state. Leakage of the rectal formulation occurred in one female rabbit (rabbit F) a few minutes after administration and, therefore, it was decided to repeat the rectal formulation and skip the oral fasted sequence. After repeating the rectal administration in this animal, leakage occurred again a few minutes after administration.

Following UPLC-MS/MS analysis the concentration of AR was found to be below the limit of detection and therefore only LF results will be presented. The relative bioavailability (F) of LF calculated from time zero to 96h time point (Liu et al., 2012), following rectal administration with respect to the oral administration dosed under fed state was **4.18** (418%). The relative bioavailability of the rectal enema with respect to the oral administration dosed under fasted state was **4.50** (450%).

The maximal plasma concentration following rectal administration was found to be 1,924 ng/mL, compared to 883 ng/mL following oral (fed) animals. The elimination rate constant, however, was comparable for all animals. The t_{max} was long for oral administration as expected or about 6 -10 hours. However, for the rectal group the t_{max} was found to be unusually long, or about 14.6 hours.

For LF, Kruskal-Wallis test showed that a significant difference ($\alpha < 0.05$) existed among treatments groups, both with respect to C_{max} and AUC. Post hoc analysis using the adjusted Holm-Bonferroni α-value revealed that the difference was only significant between the oral groups and the rectal group, but no significant difference was found

between the two oral groups. Table 45 shows the pharmacokinetic parameters of lumefantrine when dosed orally and rectally.

Table 45. The pharmacokinetic parameters of lumefantrine following oral (fasted or fed) administration of the product Lonart® 20/120 suspension and compared with rectal administration of LF. All animals received 24 mg LF per kg body weight. Significance was measured between oral (fed) animals and rectal administration.

	Rectal	Oral (fed)	Oral (fasted)	*p*-value[1]
D (mg/kg)	24	24	24	
C_{max} (ng/mL)	1,924 ± 658	883 ± 471	333 ± 301	0.012
t_{max} (h)	14.6 ± 7.2	6.0 ± 1.3	10.0 ± 8.1	0.077
V_d/F (L/kg)	14 ± 4	45 ± 48	82 ± 73	0.167
V_d (L)	272 ± 204	198 ± 195	359 ± 243	0.537
k_e (h^{-1})	0.030 ± 0.017	0.037 ± 0.014	0.044 ± 0.020	0.465
$t_{½}$ (h)	33.8 ± 25.1	24.3 ± 18.9	18.4 ± 7.7	0.475
MRT (h)	48.0 ± 31.8	31.1 ± 21.0	33.2 ± 6.5	0.306
Cl/F (L/h/kg)	0.36 ± 0.15	1.6 ± 1.9	4.6 ± 2.9	0.162
Cl(L/h/kg)	1.4 ± 1.1	1.6 ± 1.9	3.0 ± 1.7	0.840
$AUC_{0\text{-}96h}$ (ng/mL·h)	53,501 ± 26,404	16,815 ± 10,426	11,576 ± 9,986	0.008
$AUC_{0\text{-}inf}$ (ng/mL·h)	63,861 ± 37,445	17,575 ± 10,186	11,893 ± 10,087	0.027
F_{rel}	4.18 ± 2.73	(reference value)	0.58 ± 0.282[2]	

1 = comparing oral (fed) v.s. rectal administration; 2 = (n = 4) because an outlier was removed

When the median peak times were analyzed instead of average, the t_{max} was found to be 6 h (4–8 h), 6 h (4–24 h) and 10 h (10-24 h) following oral fed, oral fasted and rectal administration, respectively. No significant difference was found for the half-lives of LF.

Similarly, the median weight adjusted apparent clearance (*Cl/F*) was found to be 0.85 L/h/kg, 2.33 L/h/kg and 1.12 L/h/kg following oral fed, oral fasted, and rectal administration respectively. The median weight adjusted apparent volume of distribution (V_d/F) was found to be 27 L/kg, 71 L/kg, and 47 L/kg, for oral fed, oral

fasted and rectal administration, respectively. The median values for the mean residence time (MRT) for oral fed, oral fasted and rectal administration were 25 h, 31 h and 36 h respectively as shown in Table 46.

Table 46. Median values for selected lumefantrine pharmacokinetic parameters

	Rectal	Oral (fed)	Oral (fasted)
t_{max} (h)	10 (10 to 24)	6 (4 to 8)	6 (4 to 24)
Cl/F (L/h/kg)	1.12	0.85	2.33
V_d/F (L/kg)	47	27	71
MRT (h)	36	25	31

Figure 39 shows the average plasma concentration time curve following oral fed, oral fasted and rectal administration to rabbits. The amount of LF absorbed following rectal administration of LF in a lipophilic vehicle was found to increase the amount absorbed over 4-5-fold (up to 9.4-fold) compared to oral administration.

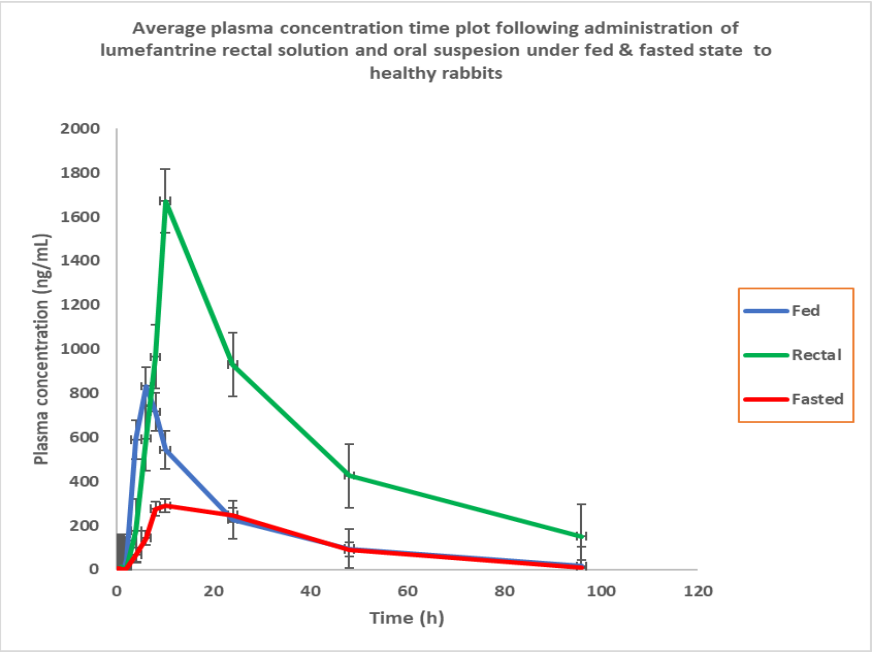

Figure 39. Mean plasma concentration time profile for lumefantrine following administration of Lonart® 20/120 to healthy rabbits (24 mg lumefantrine per kg). Blue, oral delivery to fed rabbits; red, oral delivery to fasted rabbits; and green, rectal administration. The figure was accepted for publication in paper 3.

If the bioavailability data observed in the rabbit study reflects a situation when the drug is given to human beings, then the 120 mg of lumefantrine recommended in children weighing 5 kg can be reduced to 30 mg and the plasma concentration profile may be like or close to what is seen in Figure 40. Note that the profile in Figure 40 is just an approximation as adult PK data was used in children. Getting an accurate estimate depends on accurate PkPD data from children which is lacking in literature.

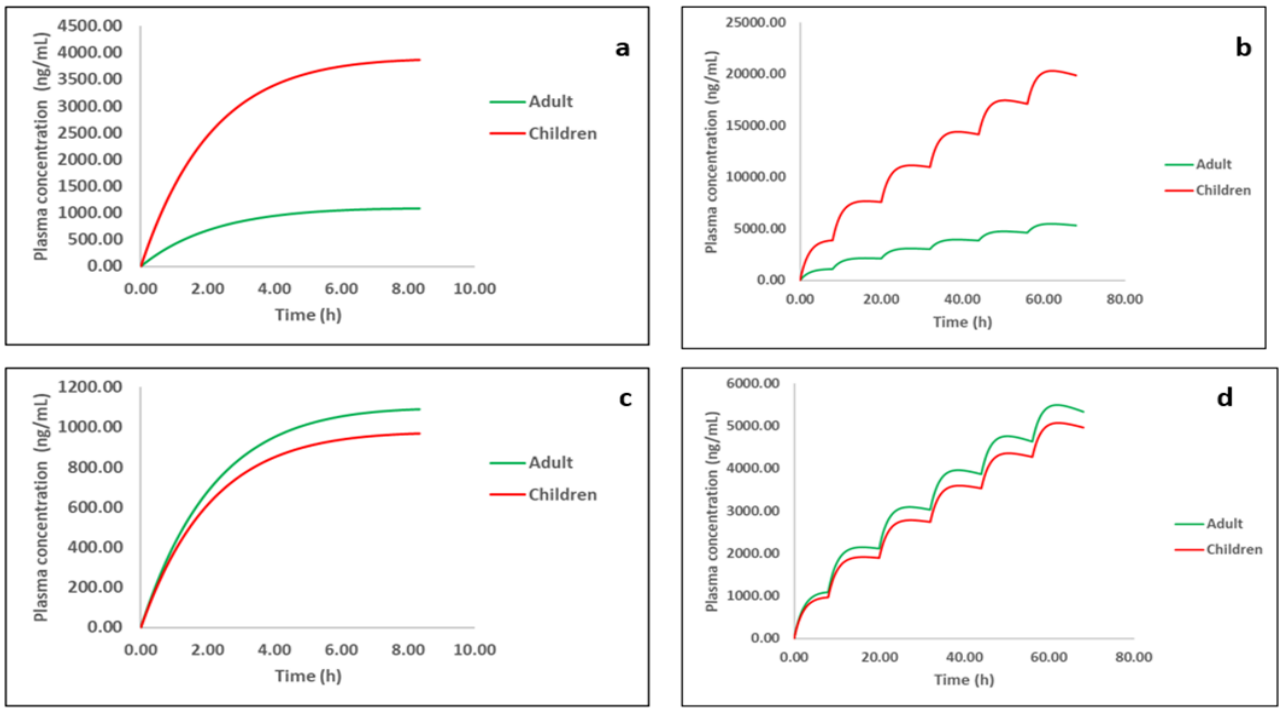

Figure 40. Plasma concentration profiles of LF when given 480 mg to adults and 120 mg to children orally as a single dose (a) and multiple doses every 12 h for 3 days (b); and when the dose in children is adjusted to 30 mg as a single dose (c) and as a multiple dose every 12 h for 3 days (d)

4.6 Estimation of pediatric dosage of antimalarial drugs, using pharmacokinetic and physiological approach

Dosing children based on traditional methods of scaling pediatric doses from adults to children showed that some children were undertreated while other children were overtreated. It was observed that children that might be prescribed AR-LF using Young's rule receive the lowest dose in comparison to those prescribed according to other methods and WHO. On the other hand, children prescribed AR-LF according to a method that is similar to allometric scaling receive the highest doses. Figures 41 and 42 show dosages of artemether and lumefantrine respectively.

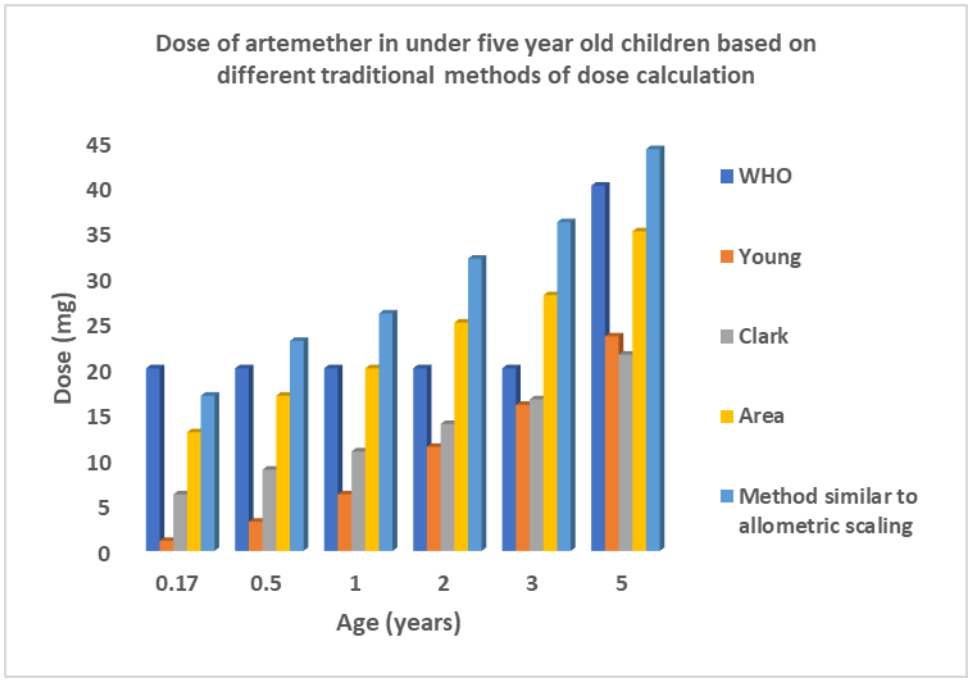

Figure 41. Doses of artemether in under five year old children based on different dose calculation methods

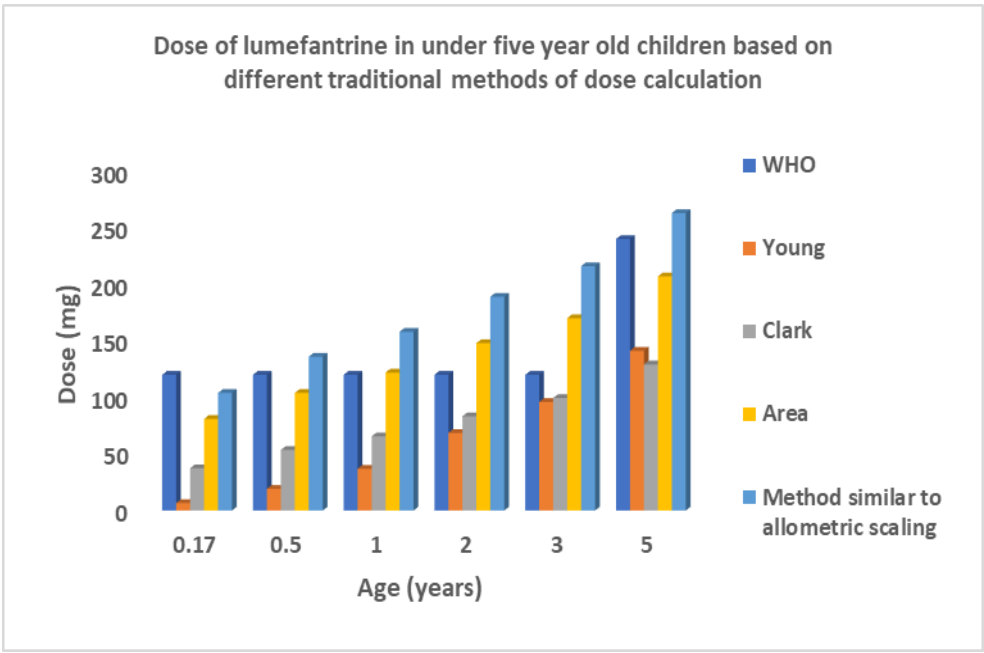

Figure 42. Doses of lumefantrine in under five year old children based on different dose calculation methods

Calculations using the pharmacokinetic data and the lowest dose for AR and LF (20 and 120 mg) respectively for infants that weigh 5 kg, the estimated PK profile showed that children were overdosed when compared with the standard dose prescribed for adults However, when a combination of the PK data, the desired plasma concentration and the physiological information such as maturation of metabolic enzymes were used in estimating the doses, a better PK profile was obtained than when using either pharmacokinetic data alone or just basing on bodyweight. It was surprising to note that malnourished children may require special adjustments in the dose for lipophilic drugs that depend on bile salts to be absorbed. The data was originally published in paper 3.

Calculations obtained on using information on liver development and maturity of enzymes responsible for metabolism showed that enzymes take time to mature as shown in Figure 43 and therefore, clearance is slow in infants and children as compared to older children.

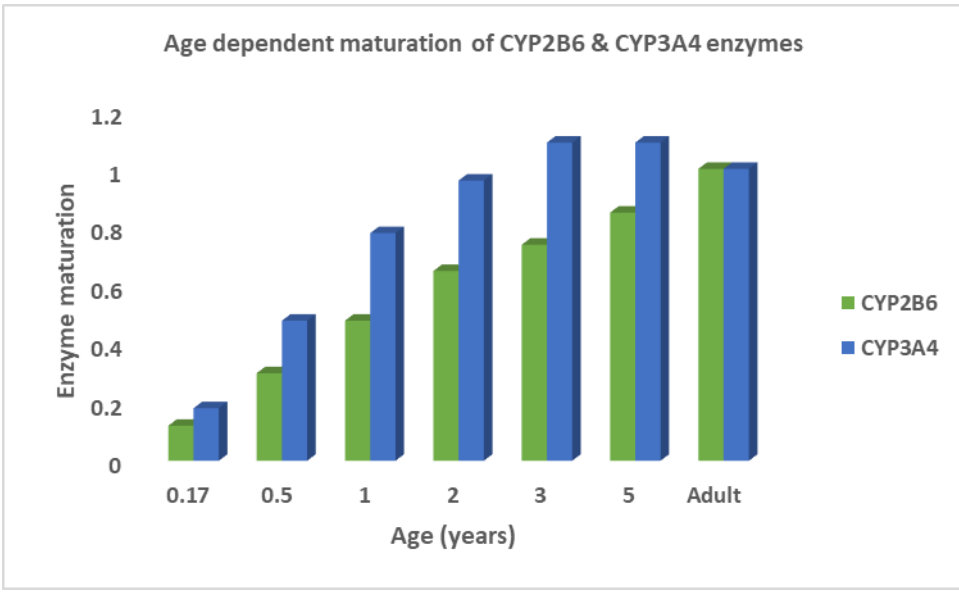

Figure 43. Age dependent maturation of CYP2B6 & CYP3A4 responsible for metabolism of artemether and lumefantrine

The results in Figure 44 revealed that clearance of AR and LF is slow in infants and children as compared to adults. The results also showed that artemether is cleared faster than lumefantrine in under five-year-old children. In addition, results showed the clearance of AR in children aged above two years old is similar to or closer to the clearance observed in adults while lumefantrine is very slowly eliminated in under five-year-old children.

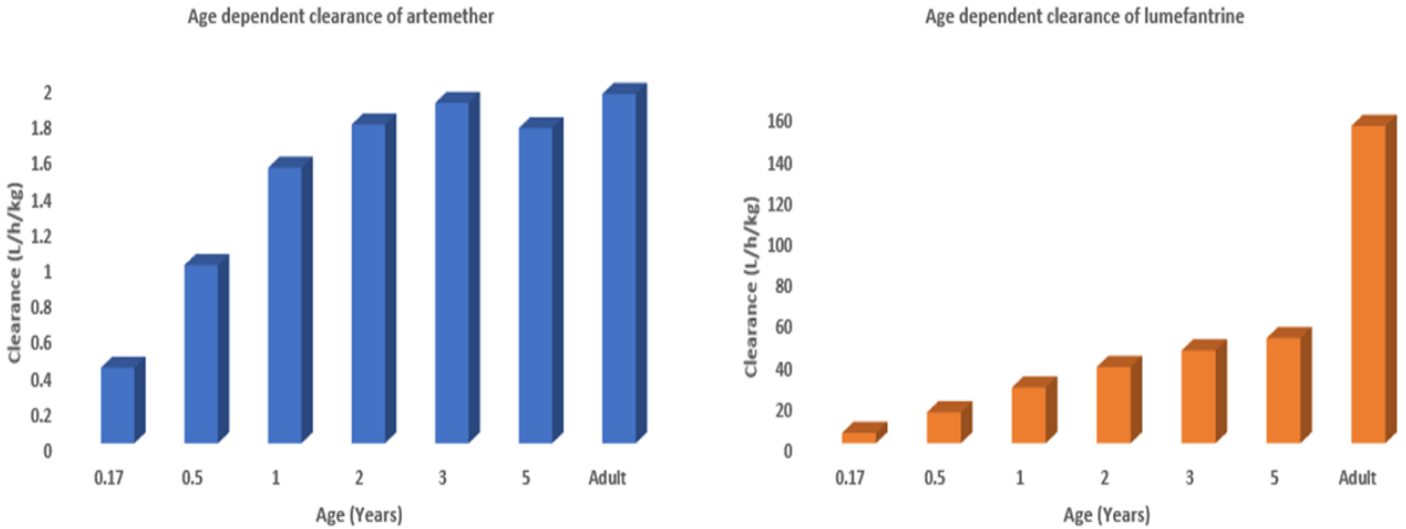

Figure 44. Age dependent clearance of artemether & lumefantrine in under five year old children in comparison to adults

5 Discussion

5.1 Preliminary screening and saturated solubility study

The main goal was to identify a suitable vehicle(s) that could dissolve and keep both AR and LF in solubilized state. Artemether dissolved in all the tested vehicles and did not precipitate when left at room temperature (21 to 23°C) for over a year. This agrees with what other authors have reported that MCTs are able to dissolve less lipophilic drugs (Savla et al., 2017) and that they have higher solvent capacity than SCTs and LCTs (Le Bars et al., 2015). Lumefantrine dissolved in the selected vehicles in very minimal amounts and precipitated shortly. However, when decanoic acid was added to samples that contained precipitated LF, LF dissolved. During saturated solubility study LF showed highest solubility in monounsaturated omega-9 fatty acid and decanoic acid. One of the possible reasons is that LF is more lipophilic than AR. The length of the fatty acid chain also has a vital role in influencing the dissolution of the drug (Savla et al., 2017). Patel and colleagues reported that the increased solubility of LF in monounsaturated omega-9 fatty acid could be due to formation of a complex between the carboxylic acid group of monounsaturated omega-9 fatty acid and the tertiary amine of LF (Patel et al., 2013). Basic drugs are also assumed to have an enhanced solubility when fatty acids are added to oils (Wyttenbach et al., 2022). It has also been previously reported that LF is able to form salts when mixed with carboxylic acids in acetone, as the acid can protonate the tertiary amine resulting in ionic forces between the ammonium and the carboxylate ions (Tomar et al., 2022). However, in the absence of acetone, the exact mechanism that makes LF more soluble in fatty acids than in MCTs is not known. Therefore, further studies are needed to verify or disproof whether these are probable mechanisms of action.

MCT is a triglyceride consisting of a glycerol backbone and three fatty acids having six to twelve carbon atoms. The two or three fatty acid chains of MCT belong to medium chain fatty acids (MCFA) (Wang et al., 2018). MCTs mainly contain caprylic (C8) and capric (C10) and small amounts of caproic (C6) and lauric (C12) fatty acids (Traul et al., 2000). MCTs have been reported to be nontoxic in acute toxicity tests done in various species of animals (Traul et al., 2000). When given orally, they are known to be safe and non-toxic (Le Bars et al., 2015; Murack & Messier, 2019) and are extensively used for total parenteral nutrition, infant formulas, and in people with malabsorption.

5.2 Incompatibility between artemether and lumefantrine

Artemether on its own showed no precipitation problems but LF displayed poor solubility and precipitated in the tested lipids. When AR and LF were mixed in MCT, precipitation occurred faster than in samples where LF was dissolved alone. The aim was therefore to find the mechanism behind LF precipitation in MCTs. It has been previously reported by other researchers that during structural analysis of LF, two C-Cl···π interactions and a few π···π interactions were observed in the crystal structure. A significant contribution of C–H···Cl interactions to the intermolecular forces of LF were also shown by Hirshfeld surface analysis (Pansuriya et al., 2019). These intermolecular forces may have a significant contribution towards the precipitation of LF on its own, but it is not clear which intermolecular forces are the major ones at play in situations where artemether precipitates out along with LF.

In this study, evidence of potential interactions between LF and MCT was revealed by NMR analysis. In addition, greater interactions were observed when a minimal amount of AR precipitate was also present in the sample. The NMR spectrum of the precipitate of LF alone in MCT, showed a small peak shift, mainly in the aromatic region where all the peaks of LF were being slightly shielded compared to the spectrum with pure LF. A greater shielding effect was observed for all the aromatic peaks in the NMR spectrum of the precipitate of AR-LF in MCT. Evidence of interactions between different molecules, e.g. between DNA and drug molecules that could act as interactions or groove binders have been previously observed by these types of NMR peak shifts (Feigon et al., 1984). Surprisingly, when AR and LF were mixed by themselves without the addition of MCTs, the peak shift was not observed. Therefore, this raises questions whether the presence of MCTs cause stronger intermolecular forces between various LF molecules in solution and/or between AR and LF. The presence of MCT vehicles seemed to be important for the peak shifts that were observed in the NMR spectra. However, the exact mechanism of interaction that led to the joint precipitation was not known and this may explain why LF is not found in solution form, such as injections. Further research is required to verify the kind of intermolecular forces behind this.

Therefore, formulating a low volume enema of 2.5 - 10 mL containing both drugs, 20 mg and 120 mg of AR and LF respectively, according to WHO guidelines was not possible without the addition of an organic acid.

5.3 Formulation preparation

5.3.1 Formulation based on monounsaturated omega-9 fatty acid

LF, in the high concentration recommended by WHO, did not dissolve in formulations that contained less than 10% oleic acid at 25°C and 43°C. The main reason is not known though it can only be assumed that the amount of oleic acid needed to interact with LF and cause it to dissolve was insufficient. However, the inclusion of less than 7%

oleic acid in the formulation at 60°C managed to dissolve LF though precipitation occurred fast (2 weeks). This may mean that LF absorbs heat in the dissolution process (endothermic) and therefore heat speeds up its dissolving process. It is also known that the solubility of most solids rises with an increase in temperature. One of the possible reasons LF precipitated could be because the solution was very concentrated (supersaturated solution) and therefore, unstable. So, when it was stored at a lower temperature (21 to 23°C), LF reverted to its stable form. Evidence of solutes precipitating from supersaturated solutions exist in literature (Hussein, 2023). LF that was dissolved in the vehicle that contained 19 to 46% oleic acid at 25 °C to 60°C did not show any signs of precipitation for more than 12 months when stored at room temperature 21 to 23°C. This means that the amount of the organic acid that was added to the formulation was enough to interact with LF and keep it in solubilized state.

5.3.2 Statistically based design of experiment using decanoic acid in the formulation

The models were valid for both LF precipitation time (Q2 of 0.99) and dissolution time (0.93) implying that the model was very good and can make good future precision predictions. The increase in decanoic acid amount (> 10 %) made the solution remain solubilized for a longer period. Decreasing both the temperature and decanoic acid amount can lengthen the dissolution time. The dissolution time can be shortened by increasing the amount of decanoic acid as shown by the square term of decanoic acid. The regression relationship between factors and responses were significant at a p value <0.05. Regarding optimization of the formulation, the sweet spot area obtained for LF precipitation time was large implying that there can be greater areas where the formulation can be optimized. This was also evidenced by the design space plot which showed that the probability of LF precipitating is greater than 50% when less than 10% decanoic acid is included in the formulation. The mechanism by which decanoic acid increased solubilization of LF may be similar to monounsaturated omega-9 fatty acid since it also possess a carboxylic acid group. Based on the preclinical studies in rabbits, a clinically relevant dose of LF may be less than the dose that was used in the experiments, which may also result in the reduction of the amount of decanoic acid needed in the formulation.

5.3.3 Formulation containing 10% of a mixture of decanoic acid and monounsaturated omega-9 fatty acid

LF dissolved in all the formulations regardless of the ratios in which decanoic and monounsaturated omega-9 fatty acids were mixed. It is also worth noting that these formulations did not contain mPEG 350 and therefore it is not known if LF would remain soluble if mPEG 350 was included. The results need to be interpreted with caution when compared to those containing a mixture of organic acids in the presence of mPEG 350.

5.3.4 Formulation containing a mixture of decanoic acid and monounsaturated omega-9 fatty acid in percentages of 1%, 5%, 10%, and 15%

Complete precipitation of LF in formulations that contained 1% and 5% of the organic fatty acids was observed in less than two weeks and less than five months respectively. However, LF remained solubilized for more than 12 months in formulations that contained 10% and 15% of the organic fatty acids. Although these formulations were not designed using MODDE®, they agree with what MODDE® predicted in the formulations that contained decanoic acid only as an organic fatty acid. This means that more than 10% of the mixture of the organic fatty acid needs to be included in the formulation for LF to remain solubilized for a long time. It is also important to note that these formulations did not contain mPEG 350. However, it is not known if the absence of mPEG 350 could have contributed to the observed results.

5.3.5 Formulation containing varying quantities of mPEG 350, decanoic acid, and MCT

After heating for a period of more than 50 min LF failed to dissolve in the formulations that contained a high amount of mPEG 350 and a small amount of decanoic acid at 35°C. This could be because the solubility of LF in mPEG 350 is very low as indicated by the results of saturated solubility study. It has also been reported that polymers have limited capacity to enhance drug solubilization when added to lipid formulations (Wyttenbach et al., 2022). It is not known if formulations could have dissolved and remained solubilized when prepared at a temperature higher than 35°C.

5.4 Quantification of artemether and lumefantrine in lipid vehicle

5.4.1 Determination of lambda max

The maximum absorbance for AR was found to be 206 nm and 218 nm for the 5 - fold diluted sample and the stock solution respectively when scanned from 190 to 400 nm. This agrees with what has been reported in literature that the structure of AR lacks chromophores and therefore absorbs UV light only in the initial wavelengths (200 to 220 nm) of the spectrum. In addition, the absorptivity of AR is much low in these areas (Cesar Ida et al., 2008). The maximum absorbance for 5 - fold diluted LF solution was found to be 234 nm and 302 nm when scanned from 190 to 400 nm and from 275 to 325 nm respectively. The stock solutions of LF showed similar absorbance at 258 nm, 276 nm, and 314 nm when scanned from 190 to 400 nm and when scanned from 275 to 325 nm showed similar absorbance at 292 nm, 298 nm, and 307 nm. Higher absorbance eradings were observed for stock solutions than diluted solutions for AR.

5.4.2 HPLC quantification methods

Several RP-HPLC methods for simultaneous determination of AR and LF in solid dosage forms have been described in literature such as (Prasanna et al., 2010; Suleman et al., 2013; Sunil et al., 2010) just to mention a few. The quantification of AR in oily injection has been described in the International Pharmacopoeia - 10th edition and other researchers (César & Pianetti, 2009; Nasir et al., 2012). The methods describing simultaneous quantification of AR-LF in oily vehicles are lacking. The International Pharmacopoeia states that AR oily injection should be diluted with the mobile phase. However, oily samples are not miscible with water and results into oil droplets or separation from the mobile phase. This was also observed by César and Pianetti (César & Pianetti, 2009). In addition, both AR and LF are insoluble in water which means diluting with the mobile phase results in a reduction in solubility of the APIs. Therefore, in this project the oily formulations were diluted with either Methanol or ACN. An exception is that formulations that contained monounsaturated omega-9 fatty acid were diluted with a mixture of ACN and MeOH. Other organic solvents like 2-propanol were found to be not suitable for diluting the formulations as during quantification the peak area of AR was greatly reduced from 15 (when ACN was the diluent) to 2 mAU when 2-propanol was used as a diluent.

During quantification of AR in AR-LF fixed dose combination tablets, César et al recommended using the standard addition method whereby AR standard solution was added to formulations to increase peak area and therefore detection. The peak area of the AR standard solution was then substracted from the peak area of AR obtained from the formulation solution. The difference was the quantity of AR originally present in the formulations (Cesar Ida et al., 2008). However, in this project the standard addition method was not used but highly concentrated samples were used to increase peak detection. The Ph. Int. also states that it is necessary to use the highly concentrated standard and sample solutions containing 10 mg/mL AR (César & Pianetti, 2009). AR absorb light in the range 200 to 220 nm (Cesar Ida et al., 2008) and is reported to have been quantified at different wavelengths with UV detection such as at 210 nm (Cesar Ida et al., 2008; Debrah et al., 2016; Hoellein & Holzgrabe, 2014), 216 nm (César & Pianetti, 2009; Nasir et al., 2012), when AR undergoes acid hydrolysis and then analyzed by UV detection at 254 nm (César & Pianetti, 2009). In this project the lambda max was found to be 218 nm and therefore, AR was quantified at this wavelength. Lumefantrine is also reported to have been analyzed at different wavelengths with UV detection such as 210 nm (Kalyankar & Kakde, 2011; Suleman et al., 2013), 235 nm (Prasanna et al., 2010), 254 nm (Arun & Arul Gnana Dhas, 2011), 303 nm (Sridhar et al., 2010), and 335 nm (da Costa César et al., 2008) et cetera. However, in this project LF was analyzed at 336 nm simply because it is only at this wavelength that excipients did not interfere with the analysis.

5.4.2.1 Isocratic elution using the Phenomenex® Luna® 5 µm C18 100 Å 250 x 4.6 mm and 0.1% TFA in methanol and 0.1% TFA in water as the mobile phase

A total of 19 experiments were designed using MODDE®. Running samples at a higher wavelength greater than 234 nm was the only factor that could separate AR from excipients. However, at this wavelength the absorbance is poor. The model showed that varying MeOH and flow rate can separate LF from excipients. Decreasing the organic content and flow rate can separate LF from excipients. Results showed that using a mobile phase composition of MeOH:water (80:20) at 0.8 mL/min could achieve some separation. However, the retention times were long, 19 min for AR and 21 min for LF. The long retention times at such a high organic content could be because MeOH has a low eluting power. Reports of long run times associated with use of MeOH as an organic modifier in Reverse phase HPLC have also been documented by other researchers (Nasir et al., 2012). In addition, MeOH gives a noisy baseline at low wavelength. Although models for both AR and LF were significant, they were not good. Due to failure to achieve separation and long run times at high organic content, ACN which has high elution power was used in subsequent experiments.

5.4.2.2 Comparing the Phenomenex columns: Luna® 5 µm C18(2) 100Å 250 x 4.6 mm and the Kinetex® 5 µm C8 100 Å 150 x 4.6 mm in separating AR and LF from the excipients using 0.1% formic acid in both acetonitrile and water

Reverse phase liquid chromatography was used. Using 0.1% formic acid in ACN and water failed to separate AR and LF from both columns. Varying the mobile phase composition, flow rate, temperature and column could achieve better retention for AR and LF from excipients. Increasing the organic content, flow rate and temperature decreased AR retention time for both columns. For LF, varying the mobile phase composition, FR and column can achieve better retention. Increasing the organic content and FR decreased the retention time for LF using both columns. LF is very poorly retained (retention time > 2 min) with both columns when the organic content and FR is increased. When using the Kinetex column, AR and LF elutes almost the same time with max separation time between the two APIs of less than 0.7 min. LF being more lipophilic and a larger molecule in comparison to AR, was expected to interact strongly with the Luna C18(2) and elute after AR. Surprisingly, with the Luna C18(2) column, LF eluted first followed by AR. This could mean that LF does not interact strongly with the matrix of the Phenomenex Luna C18(2) column. On the other hand, it is not very surprising that LF eluted first when using the Kinetex C8 column and this could be because the shorter alkyl chains of the kinetex C8 may not be very retentive to LF and therefore, LF does not interact strongly with this column (Boone & Adamec, 2016; Shen, 2023).

Separation between AR and excipients can be achieved by decreasing the organic content, flow rate and use of a suitable column. AR separation from excipients was achieved with the Luna C18(2) column only at both 218 nm and 254 nm. However, the retention time for AR is long, for instance when MeOH content is increased to 50% the retention time is 59 min and 13 min when the Luna C18(2) and Kinetex C8 columns are used respectively. AR separation is achieved at both 25 °C and 40 °C but highest separation is achieved at 25 °C. LF separation from excipients was also only possible through the Luna C18(2) column only at 218 nm at both 25 °C and 40 °C when MeOH content and FR decreased. Although separation with the Kinetex C8 column was not possible, it gave shorter or reasonable run times for AR therefore, a gradient elution was tried to find out if separation from excipients could be achieved.

5.4.2.3 Gradient elution using the Kinetex column and 0.1% formic acid in both ACN and water

Unfortunately, it was not possible to achieve separation between APIs and excipients at all the wavelengths (218 nm, 236 nm, and 254 nm) that were used at 25°C. In addition, both AR and LF eluted very close to each other and merged with solvent peaks. Therefore, the Kinetex® 5 µm C8 100 Å 150 x 4.6 mm column was considered not ideal when analyzing a fixed dose combination of AR–LF. The reason could be because LF does not interact strongly with the matrix of the column and so it appears that the APIs and excipients interact in a similar way with the matrix of the column (Shen, 2023). As a result, it was decided to try an isocratic elution using the Luna C18(2) column.

5.4.2.4 Isocratic elution using the Phenomenex® Luna® 5 µm C18 100 Å 250 x 4.6 mm and 0.1% TFA in ACN and 0.1% TFA in water as the mobile phase

A total of 19 experiments were designed by MODDE®. Three wavelengths (218 nm, 277 nm, and 336 nm) were used. Wavelength was the only significant factor and it showed that running samples at a high wavelength and high organic content at 25°C regardless of all the three flow rates that were used resulted in separation of AR from excipients. At 33 °C and 40 °C very little separation < 0.5 min was achieved. This may mean that some excipients or all excipients absorbed UV light at lower or same wavelength as AR. However, at higher wavelength AR is very poorly detected in terms of area for instance peak areas of 39, 3 and 0.2 are detected at 218 nm, 277 nm, and 336 nm respectively when 90 % organic content was used at FR of 0.8 mL/min at 25 °C. The very poor detection at high wavelength is because the maximum absorbance of AR was found to be at 218 nm from the results previously discussed. Decreasing both the organic content and FR resulted in LF separation from excipients. For instance when 65% organic content and FR of 0.8 mL/min were used, separation occurred at 218 and 336 nm. However, the highest separation occurred at 336 nm. At 336 nm and 25°C

separation was highest for excipients but run time was long as AR and LF eluted at 22 min and 23 min respectively. On the other hand, when samples were analyzed at 336 nm at 40°C, separation was also high and run time though still long but was sligtly reduced to 21 and 19 min for AR and LF respectively. The reason for long run times when the organic content was decreased could be because the increased polarity of the mobile phase increased the hydrophobic interaction between the lipophilic APIs and the non-polar stationary phase, leading to late elution of both AR and LF (Boone & Adamec, 2016). Although separation of APIs from excipients was possible at both wavelengths, generally monounsaturated omega-9 fatty acid was very poorly separated or not separated at all in many runs as it gave many peaks at lower wavelengths. Therefore, the same 19 experiments were reanalyzed by excluding monounsaturated omega-9 fatty acid.

The 19 runs were reanalyzed to separate AR and LF from excipients (decanoic acid, MCT, kolliphor® PS 20, mPEG 350). It was observed that separation from excipients was only possible at a higher wavelength, 336 nm using 90% organic content at FR of 0.8 mL at 25°C for AR. For LF, separation was also only achieved at a higher wavelength, 336 nm and decreased organic content (65% organic), FR 0.8 mL/min at both 25°C and 40°C. LF retention time was slightly reduced at 40°C (19 min) versus 22 min at 25°C. However as already discussed above, AR cannot be quantified accurately at 336 nm due to poor peak area detection and similarly LF cannot be quantified using 65% organic content as the run time is long and the shape of the peak also becomes poor. Therefore, based on the information gathered the method was optimized by doing additional experiments.

5.4.2.5 Optimization of the HPLC method

The optimizer tool suggested setpoints based on conditions and objectives of responses that were specified. The first recommendation was to run samples using 65% ACN. However, the limitation of using 65% ACN was the long run time and the very poor peaks observed when AR and LF were analyzed under this condition. The other recommendations were to run samples using 78% ACN at 336 nm. However, AR is very poorly detected at a high wavelength. Therefore, it was decided to find the best setpoint using the contour plots. Using the contour plots, 76% ACN was chosen as the organic content to be run at different flow rates, at two wavelengths (218 and 336 nm) and at two temperatures (25 and 40°C). Finally, separation was achieved for both APIs from excipients. Same mobile phase composition, 0.1% TFA in ACN / 0.1% TFA in water (76:24 % v/v) was chosen for both AR and LF. However, AR was analyzed at 218 nm, FR 1.1 mL/min at 25°C while LF was analyzed at 336 nm, 0.8 mL/min at 40°C. Although the highest separation for LF was at 25°C, 40°C was chosen to decrease the LF retention time from 12 min to 8 min (8 min at 40°C). During analysis of AR-LF formulations, it was possible to accurately quantify AR using the optimized method. However, % recovery for LF from formulations was low even though results were

reproduced. When the UV spectrophotometer was used to quantify LF from formulations at 336 nm, LF was accurately quantified. LF was also accurately quantified using the optimized method at ambient temperature when a Dionex Ultimate 3000 HPLC, Ultimate 3000 pump LPG- 3400A, Ultimate 3000 autosampler, Ultimate 3000 variable wavelength detector VWD- 3400 was used. The injection volume was 20 µL.

5.5 Bioequivalence study of lumefantrine rectal enema and the commercially available generic oral suspension. A pilot study.

After successfully developing a rectal formulation (micro enema), the preclinical study was planned and designed to evaluate the formulation in vivo. Findings from this preclinical study indicated that the relative bioavailability of LF following rectal administration was at least 4 times higher than following oral suspension. When compared to fasted animals, the bioavailability was found to be even higher or ≥6 fold, indicating that rectal administration was superior to oral delivery, and that access to food seems to be important for oral administration.

If a real clinical situation is reflected by these results, the dose of LF could be reduced significantly down to 30 mg based on WHO recommendations. However, a detailed recalculation of the clinical dose (Mhango et al., 2023) is required using physiologically based calculations before conducting clinical trials.

Even though drugs administered rectally partially bypass the first pass metabolism, this does not justify the difference reflected in the bioavailability especially when other pharmacokinetic parameters such as t_{max} are considered. The difference in bioavailability between the oral fasted and oral fed states is because orally administered LF requires bile salts to become solubilized and absorbed. More differences have been reported between fasted and fed individuals receiving LF from clinical studies (Checchi et al., 2006; Lefèvre & Thomsen, 1999; Marwa et al., 2022), though a significant difference was not observed with our data. One probable explanation could be that 2 h after the study started, the rabbits got free access to food and water, which may have aided secretion of bile salts.

A lag time is reported for LF solid dosage forms following oral absorption (Ezzet et al., 2000) since the drug must first disintegrate and become dissolved before being absorbed. The observed time to peak plasma concentration (t_{max}) following oral administration in rabbits was similar to that observed in studies conducted in human beings (6–8 h) (Djimde & Lefevre, 2009). Peak times of 6 h and 8 h, respectively, have also been observed in a bioequivalence study of two generic AR–LF products administered orally in rabbits (Kuntworbe et al., 2018). However, the rectal enema that was tested contained dissolved LF in a lipophilic vehicle. Therefore, the drug bypassed the disintegration and dissolution stages, and the absorption was anticipated to be fast.

The observed median peak time following rectal administration, however, was found to be quite long (10 h). This long peak time may partly be explained by the nature of the formulation components and the anatomical difference between rabbit rectal anatomy, compared to human rectal anatomy and need to be studied further.

The rectal mucosa of rabbits weighing about 2kg was reported to be 280 µm thick (Amiry et al., 2019) while in humans it is 150 µm (Nunes et al., 2014). Therefore, the rectal mucosa of rabbits may be more resistant to letting compounds such as LF to be transported into the systemic circulation. Furthermore, human rectal mucosa does not have much water, and the capacity and rate of transport of LF across the rectal mucosa is unknown. Therefore, the long t_{max} observed in rabbits require further studies, where the distinction between human and rabbit rectal anatomy and physiology will be studied (Hof & Bridge, 2021).

Having a bio-adhesive lipophilic formulation may permit the formulation to stick to the mucosa of the rectum, transferring the drug gradually across the rectal mucosa. This needs to be researched in more detail when human trials will be planned, or in more anatomically similar animal species.

5.6 Estimation of pediatric dosage of antimalarial drugs using pharmacokinetic and physiologic approach

Poor absorption of lipids and fats due to villous atrophy can affect the absorption of lipid soluble ACTs. Other studies have reported a 5-fold increase in the absorption of LF when taken with soy milk (low fat meal) and 16-fold increase when taken with a high fatty diet (Brody, 2018). It has been reported that a minimal amount of fat, only 36 mL of soy milk (equivalent to 1.2 g fat) was needed to cause 90% absorption (Ashley et al., 2007; White, 2014). There is limited data on pharmacokinetics of lumefantrine in children which makes it a challenge to accurately estimate the dosage in under five-year-old children. Therefore, availability of accurate and adequate information on the pharmacokinetics and pharmacodynamics of a drug is very important to estimate accurate doses for children.

In a preclinical study that was conducted in 1–2-year-old rabbits that weighed 3.75 to 5.14 kg to get some pharmacokinetic data, showed that rectal absorption was 4 times higher to the oral absorption under fed state and 6 times higher to the oral absorption under fasted state. If this data reflects a situation when the drug is given to human beings, then the 120 mg of lumefantrine recommended in children weighing 5 kg can be reduced to 30 mg to attain the plasma concentration similar to what is observed in adults.

6 Conclusions

Formulating artemether and lumefantrine into a solution or a semisolid dosage form is difficult due to their poor solubility. The research project described in this thesis identified a suitable solvent that kept both artemether and lumefantrine in solution and factors that were significant in keeping the two drugs in solution for a long time. Screening of vehicles helped in identifying potential vehicles. During preformulation studies, LF precipitated in all formulations both on its own and in the presence of AR. However, addition of fatty acids into the lipophilic formulation, made precipitated drugs to become soluble. Significant factors for the dissolution and precipitation times of lumefantrine were identified with the help of Design of Experiment software. DOE also helped in identifying the range or limits in which factors could be investigated to achieve optimal and reproducible working conditions. The addition of a fatty acid was vital to keep lumefantrine in solution, but further work is needed to prove the mechanism by which precipitation occurs. Due to limited data on the pharmacokinetics of AR and LF in the pediatric population, a pilot preclinical study was conducted using the formulated AR-LF rectal enema and a commercially available generic oral suspension. AR-LF oral suspension and rectal enema were successfully administered to healthy rabbits and compared. The bioavailability of LF was significantly higher following rectal administration, compared with the oral route. The t_{max}, however, was surprisingly long and requires further studies, particularly with respect to the differences in the anatomical and physiology between rabbit and human rectum.

This data suggests that an adjustment in the dose will be important when LF is administered via the rectal route, to receive similar plasma levels as for healthy adults. Following adjustment in the dose, the amount of fatty acid in the formulation will also be required to be adjusted. Even though the preclinical study had low power, due to the small sample size, the data present valuable information for the next step in finding a method to provide a rescue treatment for children with severe or cerebral malaria. A clinical study with high power should be conducted to give more information because accurate and effective dosing depends on accurate estimation of the pharmacokinetics and pharmacodynamics of these drugs.

6.1 Future perspectives

Further studies are required in human subjects, such as dose finding since the results show significantly increased bioavailability following rectal administration, compared to currently known pharmacokinetics following oral administration. After that a

bioequivalence study will be needed, comparing the pharmacokinetics of commercially available oral AR-LF with low dose rectal formulation. Since the rectal anatomy in rabbits are quite different from human anatomy, it may be interesting to test the formulation in other animal models to get more information on the PK of these drugs and how the anatomical and physiological differences in the rectum influences the absorption of LF.

Finally, an efficacy study during the malaria season would be a valuable trial to be conducted to verify if the estimated dose based on the dose-finding and bioequivalence study may work.

Lastly, understanding the mechanism behind the precipitation of LF in oily vehicles is something that is worth exploring, and how fatty acids such as decanoic acid is able to keep LF dissolved.

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Original Publications

Paper I

Incompatibility of antimalarial drugs: challenges in formulating combination products for malaria

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RESEARCH ARTICLE

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Incompatibility of antimalarial drugs: challenges in formulating combination products for malaria

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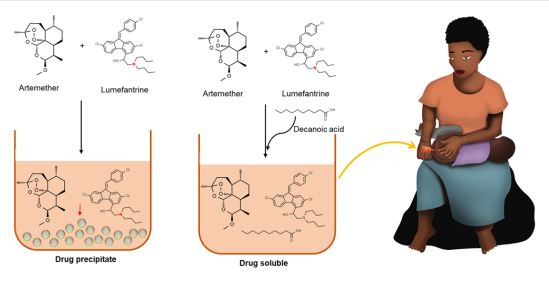
ABSTRACT

Lipophilic drugs require more advance formulation, especially if the intention is to make solutions or semisolid formulations. This also accounts for most antimalarial drugs. Although some of these antimalarial drugs are soluble in lipid vehicles, few of them, such as lumefantrine (LF), are also poorly soluble in oily vehicles. Trying to dissolve and formulate LF as a liquid formulation together with other antimalarial drugs is, therefore, a major task. When mixed in solution together with artemether (AR), precipitation occurs, sometimes with LF precipitating out on its own, and sometimes with AR precipitating out alongside LF. In this study, it was hypothesized that the use of fatty acids could lead to enhanced solubility in lipid formulation. Addition of the fatty acid solved the dissolution challenges, making LF soluble for over a year at room temperature (21–23 °C); but further research is needed to test the mechanism of action of the fatty acid. In addition, design of experiments (MODDE® 13) revealed that the amount of fatty acid in the formulation was the only significant factor for LF precipitation.

GRAPHICAL ABSTRACT



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Artemether; lumefantrine; solubility; incompatibility; malaria; fatty acids

Introduction

Immediate therapy with effective anti-malarial medicines is the major life-saving intervention in the treatment of malaria. However, this treatment is threatened by development of resistance of the malaria parasite to antimalarial drugs (Murambiwa et al., 2011). Therefore, there is a need to prevent or postpone that from happening. To treat malaria efficiently, the World Health Organization (WHO) recommends use of a combination therapy, more specifically artemisinin-based combination therapy (ACT) (Bassat et al., 2015). When malaria becomes severe or develops into cerebral malaria, the disease becomes life-threatening and every minute counts. There is a need for a simple delivery system, that can be stored in rural areas in hot tropical climate, without refrigeration, and can deliver the ACT effectively into the systemic circulation.

Artemether (AR)–lumefantrine (LF) is one of the ACTs that has been recommended by WHO to treat uncomplicated *Plasmodium falciparum* malaria (Bassat et al., 2015) on the basis that AR has fast onset of action but short half-life (2–3 hours) while LF acts slowly and has a long elimination half-life (3–6 days). Therefore, AR decreases the parasite load quite quickly and LF clears the remaining parasites. Besides this, it is not possible for the parasites to develop resistance simultaneously to both drugs as their modes of action are distinctive (Prabhu et al., 2016).

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Preformulation and liquid dosage forms are mainly based on solubilizing the drug(s) in aqueous media, and most methods used to estimate solubilization are based on numerous factors connected to aqueous solubility, such as pH, crystal structure, salt formation, etc. However, if the solubility studies are carried out in lipophilic media a different approach is required, focusing on the physical organic chemical properties of the molecule, with respect to different lipophilic solubilizers. Many antimalarial drugs are lipophilic (Aditya et al., 2010; Ma et al., 2014; Nnamani et al., 2014; Omwoyo et al., 2016). Some of these antimalarial drugs are soluble in lipid vehicles, while a few of them, such as LF are also poorly soluble in oily vehicles. Trying to dissolve LF together with other antimalarial drugs can, therefore, be a major task. When LF is mixed in a solution together with AR, precipitation occurs. This is probably one of the major reasons, why combination products in solution are not currently available (Patel et al., 2013).

Both AR and LF belong to class II drugs based on the biopharmaceutical classification system (BCS) (Patel et al., 2013; Lin et al., 2016) demonstrating their very low aqueous solubility. Generally, drugs need to be dissolved to cross mucosal barriers and be absorbed. Lipophilic vehicles may open opportunities to deliver the drugs more rapidly and extensively. Lipid based formulations have recently been classified into different classes depending on what is required from the formulation, such as type I, that only contain pure oils; type II that are classified as self-emulsifying drug delivery system (SEDDS); type III that are classified as self-microemulsifying drug delivery system, etc. (Savla et al., 2017).

The use of lipids as vehicles for the delivery of drugs with poor aqueous solubility has received more attention. Lipids have been reported to be more cost effective than polymers (Shete & Patravale, 2013; Le Bars et al., 2015). Examples of such lipids are triglycerides, that are categorized as long chain triglycerides (LCTs), medium chain triglycerides (MCTs), and short chain triglycerides (SCTs); and are used in many lipid based preparations (Le Bars et al., 2015).

MCTs consist of saturated fatty acids that have been esterified and have 6–10 carbon atoms. Hydrolysis, filtration, and re-esterification of coconut oil and palm oil produce pure MCTs (Kinsella et al., 2017; Rial et al., 2020). MCTs show higher solvent capacity than LCTs and SCTs and are less prone to oxidation (Le Bars et al., 2015; Salawi, 2022). One such lipid, called Miglyol® 812 N is a mixture of MCTs (mostly caprylic and capric acids) and has been regarded as a possible vehicle that could be used to increase the solubility of drugs (Le Bars et al., 2015).

In this work, the focus was on AR and LF. Administering AR–LF to infants and children who cannot tolerate oral medications due to severe or cerebral malaria is a challenge. In such situations, rectal drug delivery can be an option. It is important to get rapid and extensive absorption, which require that the drugs should be in a solubilized form.

Therefore, the aim of the study was to screen and identify an excipient or excipients that can solubilize and keep both AR and LF in dissolved stage.

Screening of excipients and optimization of manufacturing conditions to attain reproducible outcomes requires numerous stages and variables. The traditional approach of studying a single factor at a time while holding all others constant can be time-consuming, expensive, unpredictable, and laborious. Screening is used to detect significant factors that may influence the outcome, and to identify the limits in which they ought to be investigated. After screening, a blend of factors that can yield optimal working conditions in a process called optimization have to be identified (Benredouane et al., 2016; Mhango et al., 2017; Thorsteinsdóttir & Thorsteinsdóttir, 2021). MODDE® 13 (Sartorius, Umeå, Sweden) was used to design the experiment and perform statistical analysis.

Materials and methods

Materials

Trifluoroacetic acid, soybean oil, polyethylene glycol 400, and tetraglycol (Glycofurol) were bought from Sigma Aldrich (Gillingham, UK). Medium chain triglyceride (Labrafac Lipophile WL 1349) was kindly provided from Gattefossé SAS (Saint-Priest Cedex, France). Decanoic acid (DCA) and monounsaturated omega-9 fatty acid (oleic acid) were bought from Tokyo Chemical Industry (Zwijndrecht, Belgium). Miglyol® 812 N was kindly provided from Oleochemical (IOI) Pharma GmbH (Hamburg, Germany). Methoxy polyethylene glycol 350 (Carbowax™ Sentry™) was kindly provided from The Dow Chemical Company (Midland, UK). Extra virgin olive oil (100%) was bought from GEA (Ljutomer, Slovenia). Artemether and LF were kindly provided from Mangalam Drugs and Organics LTD (Mumbai, India). Methanol and acetonitrile were purchased from Honeywell (Seelze, Germany). The water was purified using a Milli-Q water purification system.

Methods

Preformulation studies

Initially, a pilot solubility study was conducted by mixing AR with 1 mL of the oily vehicle in a 2 mL microtube. The sample was sonicated for 2 min at 50% amplitude and stored at room temperature. Varying amounts of LF were also weighed and dissolved in 1 mL of the vehicle in a 2 mL microtube by sonicating for 2 min at 50% amplitude using Qsonica sonicators, model CL-18, serial no. 2013061001 (Cole-Parmer, Eaton Socon, UK). Lumefantrine was added in small increments followed by sonication until undissolved particles were seen. Known quantities of both AR and LF were also mixed with different vehicles and sonicated for 2 min at 50% amplitude in a 2 mL microtube.

Saturation solubility study of artemether and lumefantrine in selected lipids

The liquid lipids (1 mL) were put in a 2 mL Eppendorf tube and excess amount of drugs were added in respective tubes. Samples were mixed by vortexing on IKA® VORTEX 4 digital, model: PA1024-3I (Staufen, Germany) and thereafter allowed to shake at a speed of 250 min^{-1} for 24 hours on an Edmund Bühler GmbH (Bodelshausen, Germany) shaker at room

temperature 21–23 °C. Samples were then centrifuged at 5000 rpm for 10 min in a high-speed microcentrifuge (Corning LSE™, Vordingborg, Denmark). The supernatant was diluted appropriately with either acetonitrile or a mixture of methanol and acetonitrile. Genesys 150 UV VIS spectrophotometer (Thermo Fisher Scientific, Lillerød, Denmark) was used. High-performance liquid chromatography (HPLC) system analyses were done using a Dionex Ultimate 3000 HPLC, HPG-3400 pump with degasser, WPS 3000 TSL autosampler, TCC 3100 thermostated column compartment, photodiode array (PDA) 3000 detector (Thermo Fisher Scientific, Lillerød, Denmark) and Chromeleon chromatography workstation. Quantifications were done for LF at 336 nm (Sharma et al., 2016) and AR at 218 nm, respectively. A Phenomenex column Luna® 5 µm C18(2) 100 Å, 250 × 4.6 mm was used.

Lumefantrine solubility in solid lipids was done by melting lipids at a temperature slightly higher than the melting point of the lipid and then adding the drug in small increments. The solubility of LF in the molten lipid was determined by observing visually the presence or absence of undissolved drug (Mendes et al., 2013).

Interaction between artemether and lumefantrine

Lumefantrine was mixed with 1 mL Miglyol® 812 N and sonicated for 2 min at 50% amplitude. In a different Eppendorf tube, LF and Miglyol® 812 N were mixed first followed by sonication at 50% amplitude for 2 min. After LF was dissolved, AR was added to the solution and the sample was sonicated again for 2 min. Nuclear magnetic resonance (NMR) samples of the pure compounds (LF, AR, Miglyol® 812 N) as well as of the precipitates, both from LF alone in Miglyol® 812 N, and from a mixture of LF and AR in Miglyol® 812 N, were prepared in deuterated chloroform ($CDCl_3$) and their ^1H spectra were recorded on a Bruker Avance 400 MHz spectrometer and analyzed using MestReNova software.

Statistically based design of experiments (DoE)

A series of experiments were conducted to screen significant factors that might have influenced the main desired results. A two-level full factorial design with three factors was used, and they were put at two fixed points, i.e. at minimal or a high level. It was an interaction model, that consisted of a total of 13 runs including five center points to assess the reproducibility of the method. The experimental factors and levels used are shown in Table 1 and the outcome and their goals are used in full factorial design. Design of experiments software, MODDE® 13 from Sartorius (Umeå, Sweden), was used to design the experiments and perform the statistical analysis. Analysis of variance (ANOVA) was used to calculate the significance of the effect. The regression relationship between factors and responses was done using the *F*-test. Results were considered significant at 95% (p value <.05) significance level.

Table 1. Factors and settings (quantitative factors) used for the two-level full factorial design, where the effects of decanoic acid, Miglyol® 812 N, and temperature were studied.

Independent variables	Settings used
Decanoic acid amount (g)	0.1–1
Miglyol® 812 N (g)	1–2
Temperature (°C)	35–60

Results

Preliminary screening of lipid excipients

Artemether dissolved in all the vehicles, producing a clear solution as shown in Table 2. Lumefantrine, however, was more difficult to solubilize although it has Log *P* around 9, but it seemed to be more soluble in Miglyol® 812 N among the tested vehicles. However, precipitation occurred in all the tested vehicles. In samples containing both drugs, precipitation of LF started shortly after sonication.

Saturated solubility in selected lipids

Selected lipophilic vehicles, including Miglyol® 812 N, monounsaturated omega-9 fatty acid, DCA, Labrafac, and mPEG, were screened for further studies to identify potential lipids that could solubilize both drugs and keep them in solution. Figure 1 shows that the solubility of AR was good in all tested vehicles. LF, however, showed highest solubility in monounsaturated omega-9 fatty acid and lowest in mPEG 350. Generally, the solubility of AR in all the tested lipids was higher than that of LF. In solid lipids, LF was found to be dissolved in 30 mg/g in DCA at 60 °C.

Incompatibility between artemether and lumefantrine

When LF and AR were mixed in the same formulation, i.e. Miglyol® 812 N in the absence of DCA, precipitation occurred. NMR analysis was performed on LF by itself (Figure 2(a)), the precipitate of an AR/LF mixture in Miglyol (Figure 2(b)), AR by itself (Figure 2(c)), as well as Miglyol by itself (Figure 2(d)). When the NMR spectrum of LF by itself was compared with the precipitate from the AR/LF mixture, it could be seen that most of the precipitate was LF, but there was a small peak at 3.43 ppm, indicating the presence of some AR. Other AR peaks were either too small to be observed in the NMR or hidden behind other peaks from LF. Additionally, there was some Miglyol present in that sample as the Miglyol was not completely removed from the precipitate before analyzing it.

Another NMR comparison is shown in Figure 3, where the spectra of LF by itself (red), the precipitate of LF by itself in Miglyol (green), and the precipitate from the AR/LF mixture (blue) are overlayed. All spectra are calibrated to the $CDCl_3$ solvent peak at 7.26. When the spectra are compared, a small peak shift can be observed, especially in the aromatic region, for the LF peaks. This shift is small for the precipitate of LF by itself but increases when a slight amount of AR is present as well. Figure 4 shows an overlayed NMR spectrum of LF on its own (blue) and a mixture of LF and AR together in the absence of Miglyol (orange). The top shows a zoomed in version of the aromatic region. Both spectra were calibrated to the $CDCl_3$ solvent peak at 7.26 ppm. An NMR of an AR/LF mixture that had never been dissolved in Miglyol showed significantly less peak shift compared to LF by itself.

Table 2. Preliminary solubility of artemether and lumefantrine in different vehicles at 21–23 °C.

Drug and vehicle	HLB	Drug quantity (mg/mL)	Dissolved (Y/N)	Precipitation time
Artemether				
Miglyol® 812 N	11	60	Yes	NP
Soybean oil	7	60	Yes	NP
mPEG 350[a]	–	62	Yes	NP
PEG 400[b]	–	60	Yes	NP
Olive oil	7	10	Yes	NP
Lumefantrine				
Miglyol® 812 N	11	30	Yes	1 hour
Miglyol® 812 N	11	364	No	
Soybean oil	7	123	Yes	20 min
Soybean oil	7	248	No	
mPEG 350[a]	–	125	No	
Tetraglycol	–	90	No	
PEG 400[b]	–	73	No	
Olive oil	7	50	Yes	20 min
Artemether and lumefantrine				
Miglyol® 812 N	11	5/30	Yes	24 hours
Miglyol® 812 N + oleic acid	11	20/120	Yes	20 min
		20/120	Yes	NP
Miglyol® 812 N + decanoic acid		20/120	Yes	NP
Miglyol® 812 N	11	6.7/40	No	
Soybean oil	7	20/120	No	

NP: no precipitation.
[a]Methoxy polyethylene glycol 350.
[b]Polyethylene glycol 400.

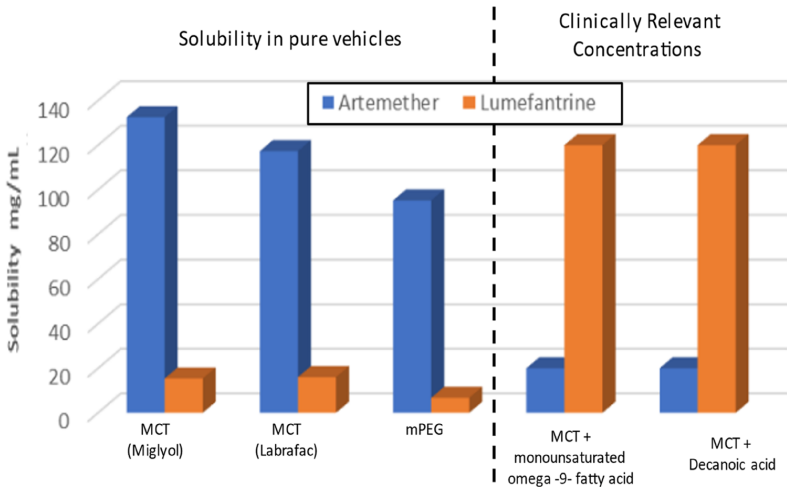

Figure 1. Solubility of artemether and lumefantrine in pure vehicles, compared to the concentrations required to achieve clinically relevant amount per mL. Two brands of medium chain triglycerides (MCT) were compared, Miglyol® 812 N and Labrafac Lipophile WL 1349. mPEG is methoxy-polyethylene glycol. For comparison, clinically relevant concentrations were achieved by using fatty acids (FA) as cosolvents, such as oleic acid or decanoic acid mixed with MCT. Note that clinically relevant amount of artemether is far from its maximum solubility in this graph, or only 20 mg/mL, where lumefantrine was soluble in a concentration of 120 mg/mL, which is significantly higher than in MCT without FA.

Adding an organic acid, such as DCA, to the formulations previously prepared as shown in Table 2, resulted in all samples dissolving.

Statistically based design of experiments

The experimental runs designed by MODDE®13 software and relative results are shown in Table 3. The amount of DCA and temperature were found to be significant factors for dissolution of LF. A decrease in DCA and/or temperature increased the dissolution time. Decanoic acid amount was found to be the only significant factor for LF precipitation as shown in Figure 5. Although, lack of significant interactions was observed, the square term of DCA was significant and it was observed that a decrease in DCA amount shortened the precipitation time. The data in Figure 6 show that the shortest dissolution time can be attained by use of high DCA amount and high temperature, where the high stability before precipitation was obtained with high DCA amount, regardless of the temperature used within the range from 35 to 60 °C. The model was valid for both LF precipitation and dissolution times as demonstrated by a Q2 of 0.99 and 0.93, respectively.

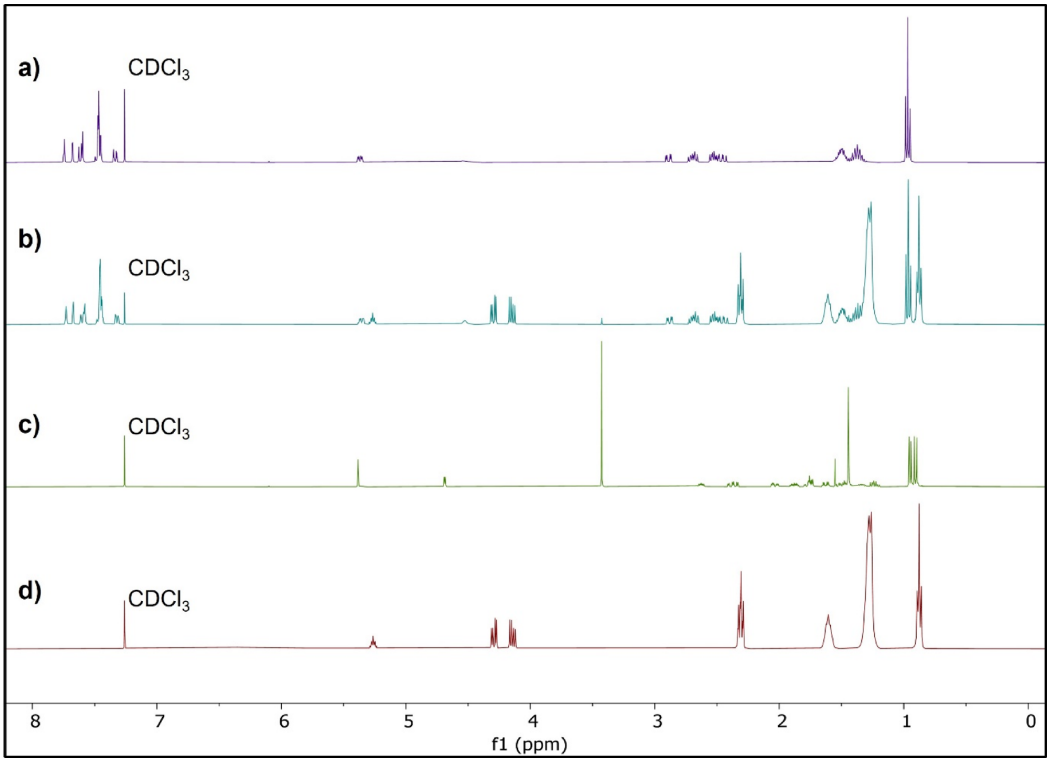

Figure 2. (a) NMR spectrum of lumefantrine on its own, (b) NMR spectrum of the precipitate that was formed from a mixture of lumefantrine and artemether, (c) NMR spectrum of artemether on its own, and (d) NMR spectrum of Miglyol® 812 N on its own.

This also shows the model was very good and therefore can make good future precision predictions. The regression relationship between factors and responses was significant at a p value <.05.

Discussion

Artemether is a methyl ether artemisinin derivative and has sesquiterpene lactone rings with an endoperoxide bridge as shown in Figure 7 and LF, formerly known as benflumetol, is a racemic fluorine derivative possessing great activity against blood schizonticides (Deen et al., 2008; Murambiwa et al., 2011). No precipitation problems were observed with AR on its own. LF, however, exhibited poor solubility and precipitation in the lipids tested.

Recent structural analysis of LF may give an insight into what the major intermolecular forces are at play for LF. Single crystal X-ray studies showed two C-Cl···π interactions and a number of π···π interactions in the crystal structure (Pansuriya et al., 2019). Hirshfeld surface analysis also showed that there was a significant contribution of C–H···Cl interactions to the intermolecular forces of LF (Pansuriya et al., 2019). These intermolecular forces, may contribute significantly toward the precipitation of LF on its own, but it is unclear, which intermolecular forces are the main ones at play in the instances where AR precipitates out alongside LF.

The NMR data showed evidence of potential interactions between LF and Miglyol or different interactions between LF when in the presence of Miglyol. Even greater interactions were observed when a small amount of AR precipitate was also in the sample. In the NMR spectrum of the precipitate of LF alone in Miglyol, a small peak shift was observed, most notably in the aromatic region where all the peaks of LF being slightly shielded compared to the spectrum with pure LF (Figure 3, red and green traces). In the NMR spectrum of the AR/LF precipitate in Miglyol, a greater shielding effect was observed for all the aromatic peaks (blue trace). These types of NMR peak shifts have previously been observed and considered evidence of interactions between different molecules, e.g. between DNA and drug molecules that could act as intercalators or groove binders (Feigon et al., 1984). Interestingly, this peak shift was not observed when AR and LF were mixed by themselves without any Miglyol present (see Supplementary Information), at least not to the same extent. This raises a question about Miglyol's role in the precipitation and on the intermolecular forces at play. If the presence of Miglyol results in stronger intermolecular forces between the different LF molecules in solution, and/or between AR and LF, especially compared to the intermolecular forces between the drug

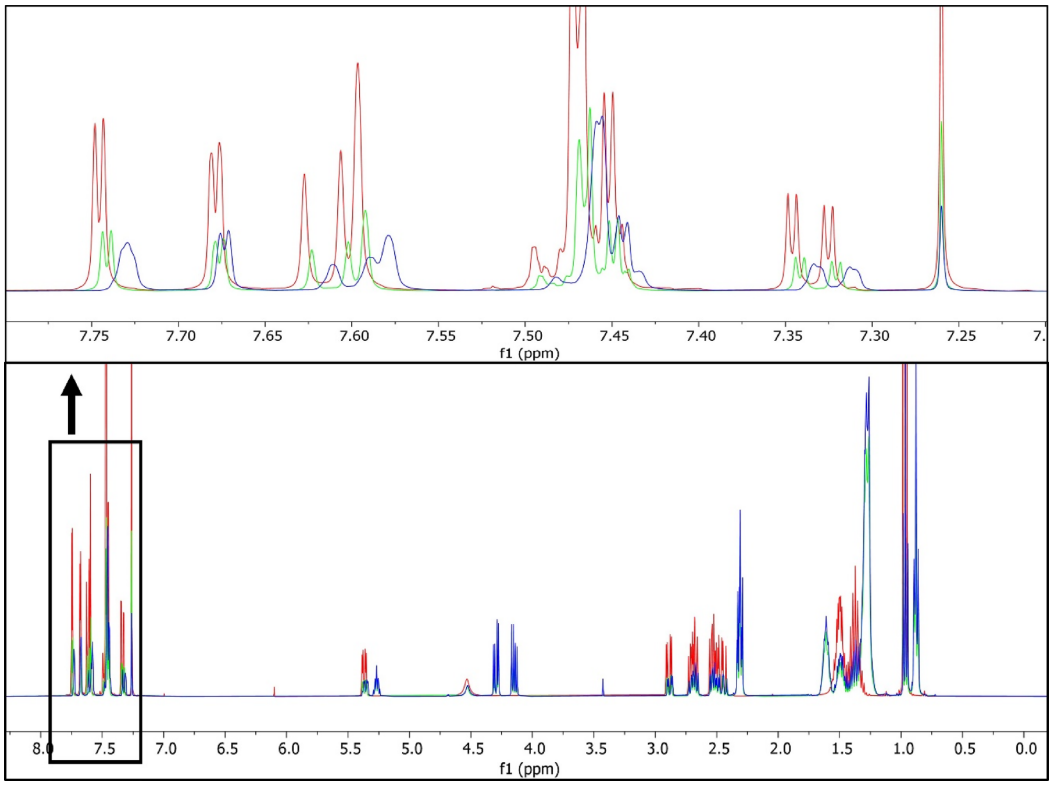

Figure 3. Overlayed NMR spectra of lumefantrine on its own (red), the precipitate formed when LF was dissolved in Miglyol® 812 N by itself (green), and the precipitate that formed from a mixture of lumefantrine and artemether in Miglyol® 812 N together (blue). The top shows a zoomed in version of the aromatic region, showing clearer peak shifts that were observed. All spectra were calibrated to the $CDCl_3$ solvent peak at 7.26 ppm.

molecules and the Miglyol solvent, that could cause these drug molecules to precipitate out together to some extent. Dissolution in the NMR solvent may break some of these interactions, but the presence of the Miglyol solvent seems to be important for these peaks shifts to be observable in the NMR spectra.

Further research is needed to verify exactly what type of intermolecular forces are at play here leading to especially the joint precipitation, but this may help explain why LF is found in solid formulations, such as tablets, capsules, and powder for oral suspension, and thus not available in solubilized liquid form, such as injections.

It was hypothesized that the use of a fatty acid might help to solubilize LF. This hypothesis was tested, initially, using a small amount of DCA as an additive to the solution mixture. When the fatty acid was added, the solubility of LF was successfully enhanced. The inclusion of a fatty acid in the formulation was found to be capable of keeping both drugs in solution, showing that this hypothesis was correct.

The mechanism of solvation is not clear at this point, but LF has shown to be able to form salts when mixed with carboxylic acids in acetone, as the acid can protonate the tertiary amine, leading to ionic forces between the ammonium and the carboxylate ions (Tomar et al., 2022). The main question remaining is how this might lead to enhanced solubility for LF in lipid formulations. One possible hypothesis includes that the strong ionic interactions may result in the aliphatic part of the carboxylate blocking other parts of LF from forming as many π···π and/or Cl···π interactions. Another potential hypothesis is that DCA could lead to formation of an inverse micelle structure that would reduce the probability of aggregation of LF by itself. More detailed physical organic chemical evidence is still needed however to verify or disproof whether these are probable mechanisms of action.

The high solubility of AR that was observed in all the tested lipids is expected as it has been reported that less lipophilic drugs can dissolve easily in MCTs (Savla et al., 2017). In addition, MCTs show higher solvent capacity than LCTs and SCTs (Le Bars et al., 2015). The moderate solubility of LF in triglycerides but its high solubility in fatty acids such as monounsaturated omega-9 fatty acids need further research to understand the mechanism that makes LF to be highly soluble in fatty acids The length of the fatty acid chain is also vital in influencing the dissolution of the drug (Savla et al., 2017).

Comparing the solubilities of the two brands of MCTs, i.e. Miglyol® 812 N and Labrafac Lipophile WL 1349, there was no difference, but Miglyol® 812 N was preferred to Labrafac on the basis that it is already being used in a commercial antimalarial product, AR intramuscular injection. Miglyol®

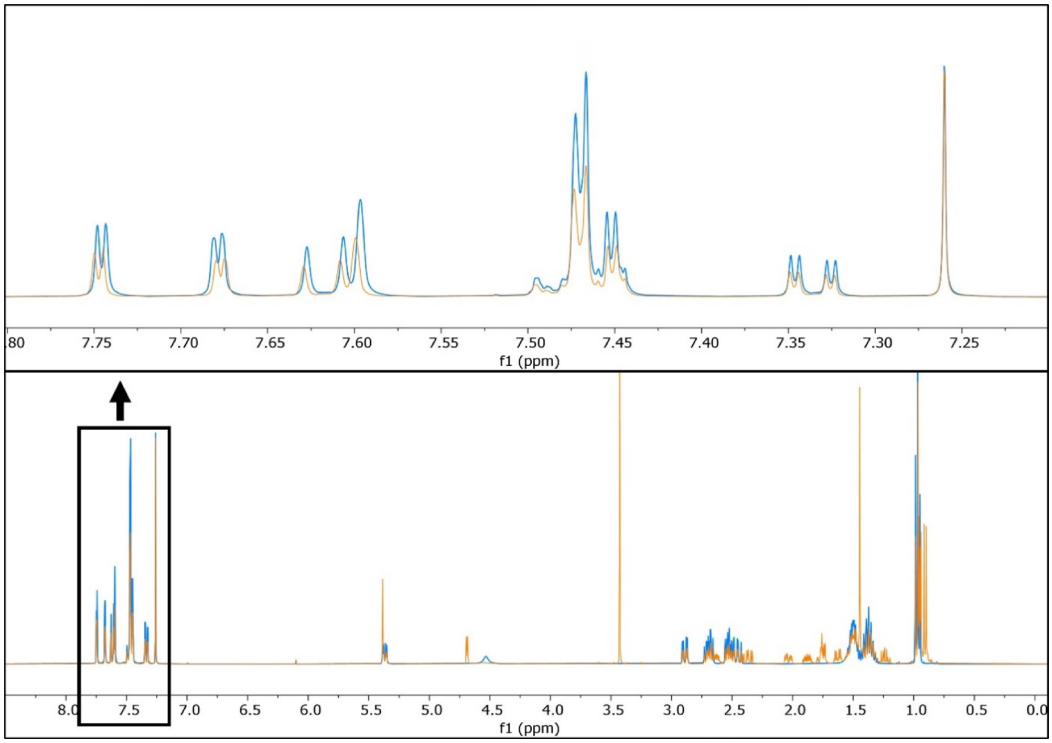

Figure 4. Overlayed NMR spectra of lumefantrine on its own (blue) and a mixture of LF and AR together in the absence of Miglyol® 812 N (orange). The top shows a zoomed in version of the aromatic region. Both spectra were calibrated to the CDCl3 solvent peak at 7.26 ppm.

Table 3. Proposed factor combination by MODDE and relative responses obtained (n = 3).

	Factors			Responses	
Run	Decanoic acid (g)	Miglyol 812® N (g)	Temperature (±°C)	Dissolution time (min)	Precipitation time (months) at 21–23 °C
1	1.0	2.0	35	36 ± 2.0	12
2	1.0	1.0	60	13 ± 1.0	12
3[a]	0.55	1.5	47.5	25 ± 1.5	12
4[a]	0.55	1.5	47.5	23.7 ± 1.5	12
5	0.1	2.0	60	39 ± 1.5	1.75
6	0.1	1.0	60	49.5 ± 2.1	0.5
7[a]	0.55	1.5	47.5	26 ± 2.0	12
8[a]	0.55	1.5	47.5	21 ± 4.0	12
9[b]	0.1	1.0	35	92	0
10[a]	0.55	1.5	47.5	19 ± 3.2	12
11	1.0	1.0	35	28.7 ± 2.9	12
12	1.0	2.0	60	16 ± 3.1	12
13[b]	0.1	2.0	35	97	0

[a]Center points.
[b]Lumefantrine did not dissolve within the specified period.

812 N was chosen as the main vehicle but since it could not solubilize the clinical dose of LF, which is 120 mg, a monounsaturated omega-9 fatty acid or/and DCA were chosen as additional excipients. More than 24% w/v DCA is needed to keep both AR and LF in solution for a period of more than a year. A temperature of up to 60 °C is needed to dissolve LF in a minimum amount of DCA (8% w/v) while increasing the amount of DCA will shorten the dissolution time and allow the solubilization process to happen at lower temperature. Medium chain triglyceride was found to be a nonsignificant factor for the precipitation and dissolution time of LF. Medium chain triglycerides are known to be safe and nontoxic, when given orally (Le Bars et al., 2015; Murack & Messier, 2019).

Mucosal surfaces contain humidity and water, even the rectal mucosa. This humidity is capable of precipitating many drugs, unless there are excipients that can bridge the gap between the lipid formulation and the humid mucosal surface. In this case, both AR and LF were dissolved in clinically relevant concentrations, using Miglyol® 812 N, a monounsaturated omega-9 fatty acid, and/or DCA. In addition, low molecular weight PEG or mPEG (methoxy polyethylene glycol) was added to the mixture for that purpose. Since this formulation could successfully solubilize both drugs, in clinically relevant doses, it will be studied further in preclinical and clinical studies.

Conclusions

The poor solubility of AR and LF makes them difficult to formulate, especially if the intention is to make a solution or a semisolid dosage form, where both drugs need to be in a dissolved form. Screening of vehicles helped identifying potential vehicles; but LF precipitated in all formulation, even in oily solution, both on its own and in the presence of AR. However, when fatty acids were mixed into the lipophilic formulation, everything became soluble. Design of experiment helped in

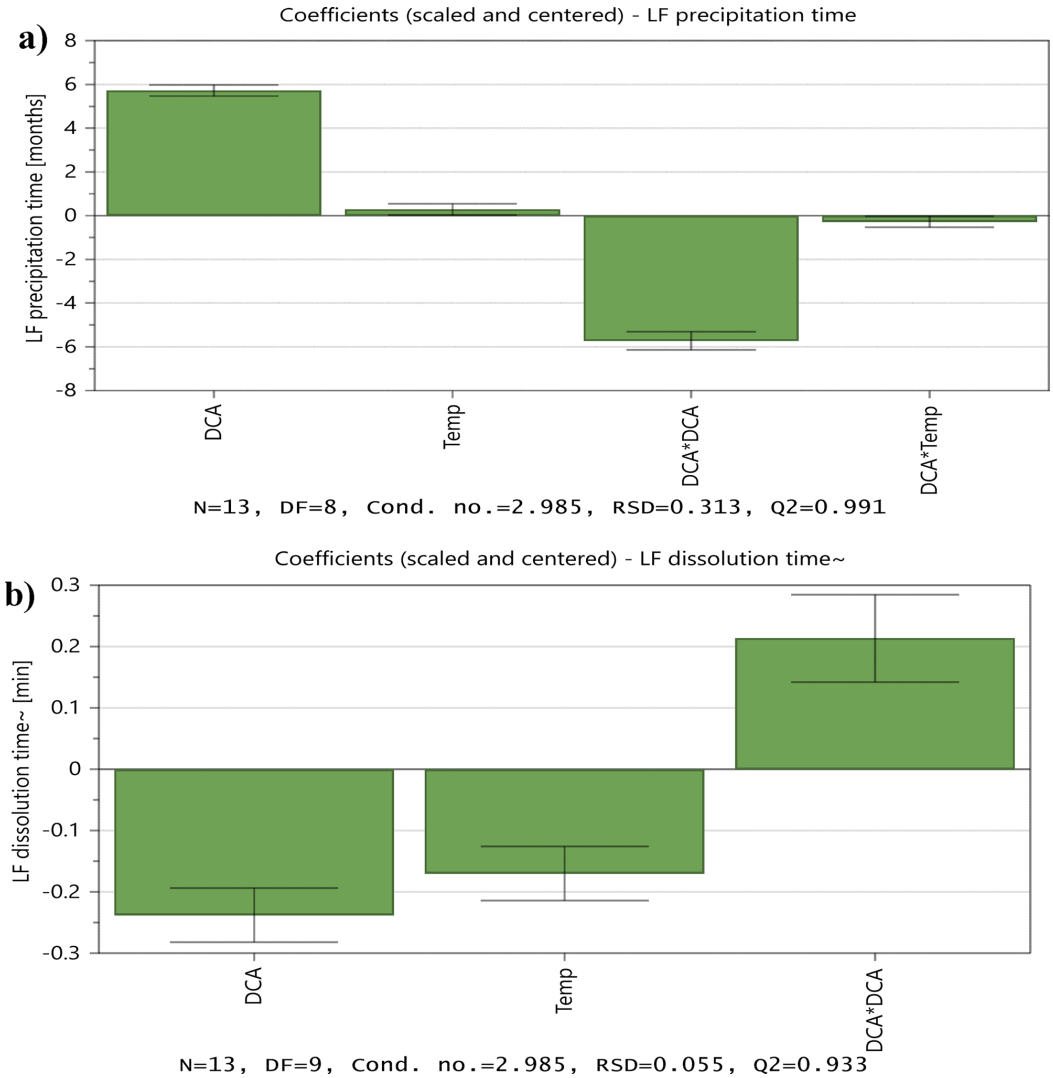

Figure 5. Coefficient plots scaled and centered for lumefantrine precipitation time (a) in months and lumefantrine dissolution time (b) in minutes.

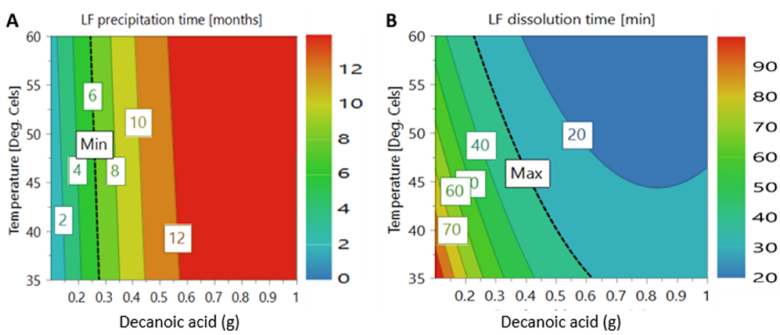

Figure 6. Response contour plots for lumefantrine (LF) precipitation time (a) and lumefantrine dissolution time (b) in a formulation containing Miglyol® 812 N. The scale shows (a) stability (in months) for lumefantrine before precipitation; and (b) time (in minutes) for solubilization of lumefantrine.

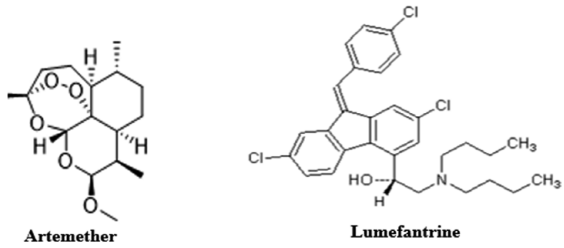

Figure 7. Structures of artemether and lumefantrine.

identifying factors that were significant for the dissolution and precipitation time of LF. It also aided in identifying the range or limits in which factors could be investigated to achieve optimal and reproducible working conditions. The inclusion of a fatty acid was necessary to keep LF in solution, but further research is needed to verify the mechanism of action.

Author contributions

Ellen K. G. Mhango: design of experiment, research, analysis, and writing the original draft. Benjamin R. Sveinbjornsson: NMR work and writing of NMR results. Bergthora S. Snorradottir: supervision on design of experiment. Sveinbjorn Gizurarson: main supervisor of the whole project.

Disclosure statement

Authors declare no conflict of interest with this manuscript.

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Data availability statement

Upon reasonable request, data can be obtained from the corresponding author.

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Paper II

Estimation of Pediatric Dosage of Antimalarial Drugs, Using Pharmacokinetic and Physiological response

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Estimation of Pediatric Dosage of Antimalarial Drugs, Using Pharmacokinetic and Physiological Approach

Ellen K. G. Mhango [1,2], Bergthora S. Snorradottir [1], Baxter H. K. Kachingwe [2], Kondwani G. H. Katundu [3] and Sveinbjorn Gizurarson [1,2,*]

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Abstract: Most of the individuals who die of malaria in sub–Saharan Africa are children. It is, therefore, important for this age group to have access to the right treatment and correct dose. Artemether—lumefantrine is one of the fixed dose combination therapies that was approved by the World Health Organization to treat malaria. However, the current recommended dose has been reported to cause underexposure or overexposure in some children. The aim of this article was, therefore, to estimate the doses that can mimic adult exposure. The availability of more and reliable pharmacokinetic data is essential to accurately estimate appropriate dosage regimens. The doses in this study were estimated using the physiological information from children and some pharmacokinetic data from adults due to the lack of pediatric pharmacokinetic data in the literature. Depending on the approach that was used to calculate the dose, the results showed that some children were underexposed, and others were overexposed. This can lead to treatment failure, toxicity, and even death. Therefore, when designing a dosage regimen, it is important to know and include the distinctions in physiology at various phases of development that influence the pharmacokinetics of various drugs in order to estimate the dose in young children. The physiology at each time point during the growth of a child may influence how the drug is absorbed, gets distributed, metabolized, and eliminated. From the results, there is a very clear need to conduct a clinical study to further verify if the suggested (i.e., 0.34 mg/kg for artemether and 6 mg/kg for lumefantrine) doses could be clinically efficacious.

Keywords: dosage; pharmacokinetics; malaria; antimalarial

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1. Introduction

Malaria is a parasitic disease that is disseminated by the bite of an infected female mosquito [1,2]. In 2019, approximately 409,000 people perished because of malaria. Approximately 67% of these were children and 94% of the cases and mortalities happened in Africa. In 2018, out of the deaths that occurred due to malaria (405,000), 94% occurred in sub–Saharan Africa [2–4]. Fever, headache, vomiting, pain in joints, and chills are the symptoms of malaria [5]. Cerebral malaria, low glucose levels, and anemia are frequent characteristics of severe malaria more noticeable in children than in grown-ups [3]. The vulnerability of children to respiratory infections, diarrhea, and other sicknesses rises when they develop recurrent malaria [3,6].

Unfortunately, most children do not survive these life-threatening afflictions. According to the World Health Organization (WHO) report in 2021, children's deaths increased by 69,000 in sub-Saharan Africa alone during the COVID-19 pandemic due to disruptions in the provision of malaria prevention, diagnosis, and treatment [7]. These life-threatening

situations require immediate treatment. Therefore, in these circumstances, it is important to have access to treatment that can be used by everyone, with the right dose for infants and small children.

1.1. Pharmacokinetics

Traditionally, dosages for children have been based on extrapolation of adult dosing using the standardized body weight of grown-ups (70 kg) or body surface area (1.73 m^2). Several methods have been suggested based on using an adult dose and correcting it using different factors based on the child's age, weight, and/or its body surface area. Examples of these equations are Young's equation, Clark's equation, Crawford's equation, etc. [8,9]. Guidelines published by the WHO on how to dose antimalarial drugs in children are also based on body weight [10]. However, children under the age of 5 years are not small adults, and their physiology, liver enzyme maturation, and serum proteins have not fully developed and may, therefore, significantly influence the pharmacokinetics of drugs and how they behave in the body.

Therefore, the use of these approaches may not provide the optimal treatment in small children and may result in either underdosing or overdosing. This is not only the case for antimalarial drugs but also for many other pediatric treatments for which the proposed dosage regimens are centered on findings from grown-up patients. The impact of underdosing on antimalarial management failure has not been recognized much [11,12]. Young children and pregnant women suffer the greatest burden of malaria. Therefore, the administered dose must be able to deliver adequate concentrations to kill the parasite, cure the child, and secure the safety of the drugs used [11].

Allometric scaling is an experimental methodology often used for scaling between animal species, where the conversion of dose is based on the surface area of the body or on the weight associated with the metabolic rate [12]. The usual purposes of interspecies scaling involve predicting the dose in humans as a result of extrapolating from preclinical experiments [10].

During development from fetus to adulthood, the human body undergoes physiological changes that affects the body's absorption, distribution, metabolism, and excretion (ADME) of both endogenous compounds as well as xenobiotics [13–15]. In oral administration of drugs, the developmental changes that influence absorption include gastric pH, gastric emptying time and motility, intestinal wall enzymes and transporters, variations in blood perfusion, the gastrointestinal microbiome, and environmental factors such as food (e.g., milk). Bile acids, biliary function, and pancreatic enzymes are essential in the absorption of lipophilic molecules, drugs, and drug esters from the GIT through solubilization and cleavage of prodrugs [15,16]. The amount and composition of bile acids are low in neonates and continue to increase during development to reach adult levels [16–18].

In relation to comorbidities, the drugs used to treat comorbidities may alter the pharmacokinetics of AR-LF or cause adverse drug reactions; for example, ketoconazole (an antifungal drug) has been reported to increase the plasma concentration of AR-LF through inhibition of the enzyme (CYP3A44) responsible for metabolizing these drugs [19]. In individuals coinfected with malaria and HIV, lopinavir/ritonavir raises AR exposure but decreases LF exposure [20]. Efavirenz also decreases AR-LF exposure, while nevirapine reduces exposure of AR but LF remains unaffected. However, AR-LF decreases exposure of nevirapine [21]. Coadministration of AR-LF with other antimalarial drugs, such as quinine, decreases the exposure of AR but has no effect on LF [22]. Meanwhile, the antimalarial mefloquine does not alter the PK of AR, but it decreases the plasma concentration of LF [23].

Rifampicin, a drug used in tuberculosis treatment, decreases plasma concentrations of AR-LF [24]. Foods such as grape juice and grapefruit raise the concentration of AR-LF [25]. These examples of drug—drug interactions or food—drug interactions may elevate the risk of toxicity or therapy failure and development of resistance.

1.2. Plasma Proteins

Many drugs bind to plasma proteins to a certain degree. Drug distribution and elimination are altered by plasma protein binding, which is influenced by different factors such as the concentration of proteins in the body, affinities between drugs and proteins, hepatic or renal diseases, and the physicochemical properties of the drug. Albumin, α_1-acid glycoprotein (AAG), and lipoproteins are the main proteins to which drugs bind [26]. Plasma albumin is the key protein (35–50 g/L), as shown in Table 1, and is responsible for binding many acidic (anionic) and basic drugs. Since the synthesis of albumin takes place in the liver, its concentration may be lowered when the liver is suffering due to disease, helminthics, or conditions such as malnutrition [26,27].

Table 1. The concentrations of selected plasma components in different population groups.

Plasma Component	Adults	Newborn	Infant	Child	Malnourished Children	Drug Binding
Plasma albumin (g/L)	35–50	29–54	44–54	40–58	↓↓ (<24.5)	Artemether
α_1-glycoprotein (g/L)	1.0–4.0	1.0–3.0	2.0–4.0	1.0–4.0	↑↑	Artemether
Cholesterol (mmol/L)	<5.2	1.9	2.3–3.4	3.4–4.4	↓↓	
LDL (mmol/L)	1.55–4.14	0.78	2.07	2.5	↓↓	Lumefantrine
HDL (mmol/L)	1.52–4.05	1.6	2.6	3.4	↓↓	Lumefantrine
VLDL (mmol/L)	0.1–1.7	0.28	0.62	0.45	↑↑	Lumefantrine
Triglycerides (mmol/L)	0.44–2.09	0.46	0.05–0.46	0.88–1.56	↑↑	Lumefantrine
RBCs ($\times 10^{12}$/L)	4.0–6.0	4.8–7.2		3.8–5.5	↑↑	Artemether/lumefantrine

↑↑ = increased plasma component, ↓↓ = decreased plasma component.

Although AAG is present in much low concentrations (1–3 g/L, see Table 1), it is responsible for binding to many basic (cationic) and neutral drugs. Like albumin, the synthesis of AAG takes place in the liver. Contrary to albumin, medical conditions such as malaria causes AAG concentrations to elevate [26,28]. Likewise, the synthesis of lipoproteins, which comprise high density lipoproteins (HDL), low density lipoproteins (LDL), and very low-density lipoproteins (VLDL), takes place in the liver and intestinal mucosa (Table 1). They are known to bind to very lipophilic basic and neutral drugs [26]. Values used in Table 1 for lipoproteins were extracted from Bogulasa heart study [29], values for malnourished children were extracted from Barroso et al. [30] and values for serum lipids were extracted from Carvajal et al. [31].

Children are known to have lower concentrations of plasma proteins, as shown in Table 1. This may affect the tissue distribution of drugs, the free (unbound) drug concentration, as well as influence the clearance. Some drugs are known to bind to other plasma components, such as lipoproteins and red blood cells, and their uptake from plasma is usually concentration dependent. Since the malaria parasite hides inside red blood cells, this could be beneficial for the efficacy of the drug. However, having a high free drug concentration may have its drawbacks, such as altered volume of distribution, clearance, and half-life, since free drug is cleared more easily than drug bound to plasma proteins and red blood cells.

The WHO recommended the use of AR and LF as one of the artemisinin-based combination therapies (ACTs) to treat uncomplicated *Plasmodium falciparum* malaria [32]. Therefore, AR and LF were chosen as the model drugs in this work. Artemether works by forming free radicles that delay the growth of proteins during development of the parasites, while LF works by detoxifying hemoglobin in the parasite [33,34]. Lumefantrine is a very highly protein bound drug (99.7%), leaving only 0.3% free concentration of active drug [35]. It binds primarily to HDL (77%), LDL (7.3%), and VLDL (6.6%). The fraction of LF that binds to albumin and AAG is negligible. The fraction that binds to red blood cells is only 8% [36].

The magnitude of protein binding may not be affected by the condition the disease (malaria) causes. LF is most likely metabolized to a low degree in vivo, indicating that it can be categorized as a capacity-limited (poorly extracted) binding-sensitive drug (i.e., a

drug that is highly bound with a low hepatic extraction ratio) [37]. AR is also highly bound to proteins (95.4%) and has, therefore, a much higher free concentration (4.6%) [35]. AR binds mainly to AAG (33%), albumin (17%), HDL (12%), LDL (9.3%), and VLDL (12%). The fraction that binds to red blood cells is approximately 11% [36].

1.3. Metabolism

Although metabolism can take place in various areas of the body, such as the kidney, lungs, gastrointestinal tract, and placenta, the liver is the main site of metabolism, and the expression of enzymes is different among infants, children, and grown-ups. Because of this, metabolism varies with age. Therefore, it is challenging to predict safe and effective pediatric drug dose using data from adults only [8,38]. Another factor that needs to be taken into consideration is that the size of the liver in infants and small children is relatively larger than that in adults. This may be reflected by variations in the activity of metabolizing enzymes with respect to liver size. Having a relatively larger organ will require relatively more blood flow, which will affect the disposition and can lead to either underexposure and treatment failure or overexposure with accompanying toxicity [39].

1.4. Nutritional Status

The relationship between undernutrition and malaria remains complex, with other links deemed controversial and others in the course of being delineated [40,41]. Overall, however, undernutrition in children under five years increases the risk of malaria, other infections, and recurrent malaria parasitemia [42–45]. Moreover, chronic undernutrition is a determinant of severe malaria complications [40,46] and being underweight contributes to approximately double the risk of malaria re-infection following successful treatment [47,48]. The effect of malnutrition on malaria and its treatment is attributed to the pathophysiology driven by malnutrition.

Severe acute malnutrition (SAM) results in pathophysiological alterations in tissue, organs, and the body in general [49]. Various physiologic adaptations in SAM include growth restriction, loss of fat, muscle, and visceral mass, reduced basal metabolic rate, and reduced total energy expenditure [50,51]. Organ systems are also variably impaired in SAM, including the immune system, in which cellular immunity is impaired and the body is rendered susceptible to infections [50,51]. Chronic undernutrition may increase the risk of severe malaria and re-infection due to the altered immune response of the host [40]. Undernutrition may also obscure the overt signs and symptoms of clinical malaria usually observed in children with a normal nutrition status [40,41].

Another significant pathophysiological alteration in malnourished children is intestinal villous atrophy in the jejunal mucosa, with resultant malabsorption of nutrients and drug substances [49]. Figure 1 shows the anatomical alterations in the intestinal mucosa as a result of severe malnutrition and the interaction with bile stimulation differentiating the responses between the two anatomically different mucosae.

The purpose of this article was to estimate the dosage for the two antimalarial drugs, artemether and lumefantrine, in young children under 5 years of age using a physiological and pharmacokinetic approach.

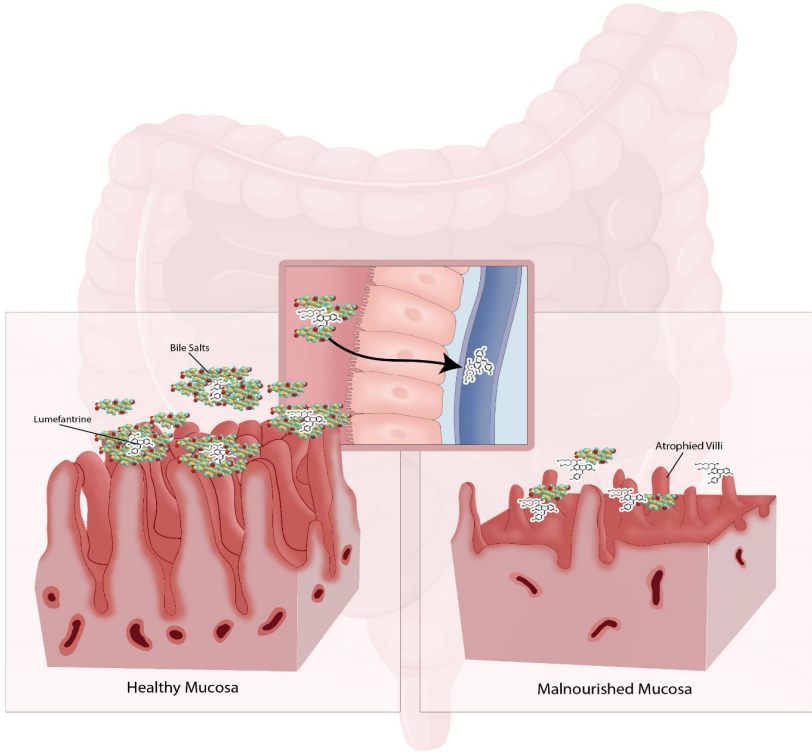

Figure 1. The intestinal mucosae in healthy and malnourished subjects, showing the atrophy of villi in the jejunum as well as the reduced amount of bile, which is required to augment the absorption of compounds such as lumefantrine.

2. Methods

2.1. Protein Binding

There are many physiological and anatomical factors that undergo transformation when newborns become infants and then grow to become children. Some of these factors are not fully developed until children are around 18 years and many of them will influence the pharmacokinetics of drugs. Predicting the free drug fraction in neonates and infants has been studied by McNamara and Alcorn [52]. Knowing the value of the unbound drug fraction in plasma (f_u) can be used to predict the free fraction in infants and children [1], as shown in Equation (1). The unbound free fraction of drugs in children (fu_{child}) can be estimated from the free fraction in adults (fu_{adult}) and the protein concentration [P] ratios between adults and children, using Equation (1) [1].

$$fu_{child} = \frac{1}{1 + \frac{[P]_{child}}{[P]_{adult}} \times \frac{(1-fu_{adult})}{fu_{adult}}} \quad (1)$$

2.2. Metabolism and CYP Enzyme Maturation

The maturation of enzymes takes time and has been expressed as the ratio of adult abundance based on age. The age-related maturation of enzymes that are important for artemether (CYP2B6 and CYP3A4) and lumefantrine (CYP3A4) is expressed as [53]:

$$\text{CYP2B6} = \frac{1.07 \times Age}{1.13 + Age} \quad (2)$$

For the enzyme CYP3A4, the maturation rate changes around 2.3 years, so for children below the age of 2.3 years, the following maturation model should be used [54]:

$$\text{CYP3A4} = 0.11 + \frac{0.95 \times Age^{1.91}}{0.64^{1.91} + Age^{1.91}} \quad (3)$$

Then, for children 2.3 years and older, the maturation rate follows [54]:

$$\text{CYP3A4} = 1.1 - 0.123 \times e^{(-0.05(Age-2.2))} \quad (4)$$

Using the enzyme maturation model incorporating the enzymes involved for each compound, such as artemether, the overall degree of enzyme maturation (MFA) in children below the age of 25 years is calculated by adding the outcomes into the following equation [8], such as for artemether [54]:

$$MFA = (0.111 \times CYP2B6) + (0.889 \times CYP3A4) \quad (5)$$

This information may then be used to calculate the estimated clearance in children using the following equation. Note that this equation does not account for differences in the volume of distribution in children compared to that in adults [54]:

$$Cl_{children} = Cl_{adults}\left(\frac{Liver\ weight(child)}{Liver\ weight(adult)}\right)^{0.75} \times MFA \quad (6)$$

2.3. Dosage Calculation

The equations that can be used to extrapolate the adult dosage to an equivalent dose in children are shown below.

2.3.1. Young's Rule

Young described the relationship between adult and child doses using the following equation, in which he only focused on the child's age [55]:

$$D_{child} = average\ adult\ dose\left(\frac{age\ of\ child(y)}{age\ of\ child(y) + 12}\right) \quad (7)$$

where D_{child} is the dosage in a child.

2.3.2. Clark's Rule

Clark described the relationship between adult and child doses using the following equation, in which he only focused on the weight of the child and compared that with the equivalent dose in a 68 kg adult [55]:

$$D_{child} = adult\ dose\left(\frac{weight\ of\ child(\text{kg})}{68\ \text{kg}}\right) \quad (8)$$

2.3.3. Area Rule

Few researchers have focused on the body surface area (BSA) as the method to calculate the dose [55]:

$$D_{child} = adult\ dose\left(\frac{BSA\ of\ child(\text{m}^2)}{1.73\ \text{m}^{2*}}\right) \quad (9)$$

Here, the BSA can be calculated using the Mosteller formula, where W is the weight in kg and H is the height in cm [56]:

$$BSA = \sqrt{H \times \frac{W}{3600}} \quad (10)$$

2.3.4. Other Method Similar to Allometric Method

Another method that has been used by some researchers is similar to allometric scaling, in which the adult weight is proportional to the child weight to the 0.75 power [57], as shown below:

$$D_{child} = adult\ dose \times 1.5\left(\frac{weight\ of\ child\ (\mathrm{kg})}{70\ \mathrm{kg}}\right)^{\frac{3}{4}} \quad (11)$$

A variation of this method was introduced by Mahmood [58], who suggested the power varies depending on the child's age. This author used the following formula to estimate the clearance in children:

$$Cl_{pediatric} = Cl_{adult} \times \left(\frac{weight_{pediatric}}{weight_{adult}}\right)^{exponent} \quad (12)$$

where the exponents are 0.75 for children older than 5 years, 0.9 for children 2–5 years, 1.0 for infants 3 months to 2 years, and 1.2 for infants 1–3 months.

2.4. Pharmacokinetic Method

The pharmacokinetic parameters for artemether and lumefantrine listed in Table 2, were obtained from WHO and values for children and malnourished children were extracted from different articles [19,59]. When the pharmacokinetic method is used, the dose is calculated based on the desired plasma concentration. Here, the desired concentration may be based on the plasma concentration reached when an adult receives a dose. At any time, point, the plasma concentration (C_p) in an adult may be calculated using adult dose and pharmacokinetic parameters for these antimalarial drugs in adults, using the following equation:

$$C_p = \frac{FDk_a}{V_d(k_a - k_e)}\left(e^{-k_e(t-t_0)} - e^{-k_a(t-t_0)}\right) \quad (13)$$

where F is the fraction of dose absorbed, V_d is the volume of distribution, k_a and k_e are the absorption rate constant and elimination rate constant, respectively, t is the time of sampling, and t_0 is the lag time. Then, if the C_p found in adults is used and the pharmacokinetic parameters found in children (marked with′, such as F') are added, the appropriate dose in children can be estimated.

$$D_{child} = \frac{C_p V'_d(k'_a - k'_e)}{F'k'_a\left(e^{-k'_e t} - e^{-k'_a t}\right)} \quad (14)$$

Table 2. The pharmacokinetic parameters for artemether and lumefantrine, based on values obtained from the World Health Organization (Guidelines for treatment of malaria, 3rd edition) and literature.

	Artemether			**Lumefantrine**			
Parameter	**Adults**	**Children**	**Malnourished Children**	**Adults**	**Children**	**Malnourished Children**	**Comments**
Dose (mg/kg)	1.14	5		6.86	29		
C_{max} (ng/mL)	100	119		7.91 **		15% lower	Malaria (and food) increases C_{max} for AR 2-3 fold
t_{max} (h)	2.0			6			
$t_{1/2}$ (h)	1.9	16		95	123	12% shorter	Malaria makes half life significantly longer (LF)
AUC (ng·h/mL)	320	392		207		31% lower	Malaria increases AUC significantly (AR)
Vd (L/kg)	6.05	0.9		3.8/0.71	1.3		
Cl (L/h per kg)	0.91						3 fold lower Cl in <3 months vsv >3 months (AR)
k_a (/h)	1.52	1.2		0.44	0.46		Malaria lowers k_a significantly (LF)

Table 2. *Cont.*

	Artemether			Lumefantrine			
Parameter	**Adults**	**Children**	**Malnourished Children**	**Adults**	**Children**	**Malnourished Children**	**Comments**
F (%)	43			65			Lower in fasted subjects, or age <5 year (LF)
fu (%)	4.6			0.3			
Drug Interactions							
Effect of ketoconazole	↑			↑			
Effect of mefloquine	NE			↓			
Effect of quinine	↓			NE			
Effect of lopinavir/ritonavir	↓			↑			

** Food may increase the absorption of lumefantrine up to 16—fold due to increase in bile salts. Units for LF are (µg/mL). NE = No effect, AUC = Area Under the Curve, V_d = volume of distribution, t_{max} = Time to maximum plasma concentration, Cl = Clearance, ↑ = increased plasma concentration or exposure, ↓ = decreased plasma concentration or exposure.

2.5. Physiologically Based Pharmacokinetic Calaculations

Using the information available in the literature, enzyme maturation and other parameters can be calculated that may help in calculating the clearance in children.

$$Cl_{children} = Cl_{adults}\left(\frac{Weight}{70\ \text{kg}}\right)^{0.75} \times MFA \quad (15)$$

3. Results

3.1. Metabolism and CYP Enzyme Maturation

Calculated estimations or theoretical clearance rates of artemether and lumefantrine based on CYP enzyme maturation and liver development are presented in Table 3. The tissue water/fat ratio is not included in this calculation, but tissue distribution and tissue elimination have a significant influence on the clearance, which is not expressed in this table. Standard liver volume was calculated based on Walter et al. [60], and enzyme maturation was calculated based on Bonate et al. [54] and Johnson et al. [53].

Table 3. Theoretical calculation of clearance for lumefantrine and artemether based on liver enzyme maturation in children, using the elimination rate constant in adults as a reference value.

	Standard Liver	Liver Volume	Enzyme Maturation		Lumefantrine	Artemether
Age	**Volume (mL)**	**As % weight**	**CYP3A4**	**CYP2B6**	**Clearance (L/h/kg)**	**Clearance (L/h/kg)**
2 mo	162	3.06	0.18	0.12	0.42	5
6 mo	198	2.60	0.48	0.30	0.99	15
1 y	223	2.40	0.78	0.48	1.53	27
2 y	258	2.19	0.96	0.65	1.77	37
3 y	287	2.03	1.09	0.74	1.89	45
5 y	334	1.82	1.09	0.85	1.75	51
Adult	1.648	2.35	1.00	1.00	1.94	154

3.2. Dosage Calculation

Table 4 shows the calculated dose based on different scaling methods for artemether and lumefantrine in healthy children from 2 months to 5 years.

3.3. Pharmacokinetic Method

According to the guidelines published by the WHO (WHO, 2015), the recommended dosage for artemether is 5–24 mg/kg but 29–144 mg/kg for lumefantrine. Since artemether and lumefantrine tablets are fixed solid dosage forms, dosing is per both the WHO and manufacturer guidelines. Infants and children weighing 5 kg to less than 15 kg receive 20 + 120 mg of artemether—lumefantrine. Children weighing 15 kg to less than 25 kg receive 40 + 240 mg of artemether—lumefantrine twice a day for three days. The second dose is given after 8 h and then every 12 h for a total number of three days. Using the lowest dose

(20 and 120 mg/kg for artemether and lumefantrine, respectively) for an infant weighing 5 kg, the estimated pharmacokinetic profile for these two drugs may be seen in Figure 2 and compared with the standard dose for adults.

Table 4. Calculated dosages for children, at different ages, using various published methods to extrapolate from adult dose to pediatric dose, compared to WHO recommendations for the same age.

Artemether (mg)							
Age	**Weight (kg)**	**Height (cm)**	**WHO**	**Young's Rule**	**Clark's Rule**	**Area Rule**	**Allometric Scaling**
2 mo	5.3	57.3	20	1.1	6.2	13	17
6 mo	7.6	66.6	20	3.2	8.9	17	23
1 y	9.3	75	20	6.2	10.9	20	26
2 y	11.8	87.3	20	11.4	13.9	25	32
3 y	14.1	95.5	20	16.0	16.6	28	36
5 y	18.3	109.8	40	23.5	21.5	35	44
Lumefantrine (mg)							
Age	**Weight (kg)**	**Height (cm)**	**WHO**	**Young's Rule**	**Clark's Rule**	**Area Rule**	**Allometric Scaling**
2 mo	5.3	57.3	120	6.6	37.4	81	104
6 mo	7.6	66.6	120	19.2	53.6	104	136
1 y	9.3	75	120	36.9	65.6	122	158
2 y	11.8	87.3	120	68.6	83.3	148	189
3 y	14.1	95.5	120	96.0	99.5	170	216
5 y	18.3	109.8	240	141.2	129.2	207	263

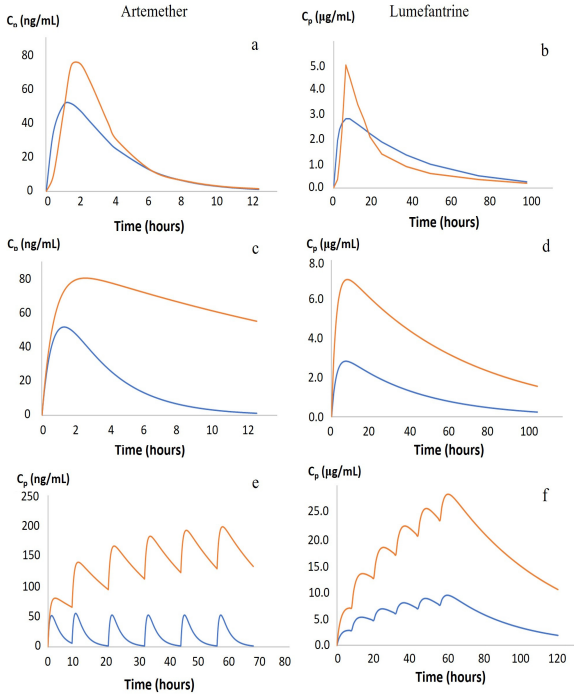

Figure 2. Plasma concentration profiles of artemether and lumefantrine. (**a**,**b**) The calculated pharmacokinetics in adults (blue) in comparison with real adult data based on the pharmacokinetics of Coartem®, from Novartis (Basel, Switzerland) (orange). (**c**–**f**) The pharmacokinetics in adults (blue) and a 5 kg child (orange), using pharmacokinetic values from the literature and dosed according to the guidelines from the World Health Organization. (**c**,**d**) A single dose administration of artemether (**c**), and lumefantrine (**d**). (**e**,**f**) The appearance of full treatment when doses are administered at time 0, 8 h, then every 12 h for 3 days for artemether (**e**), and lumefantrine (**f**).

3.4. Physiological Information

Using physiological information to estimate the dose, including the maturation of metabolic enzymes and the pharmacokinetics of the drug, the estimated doses of artemether and lumefantrine to be used in children are suggested and compared with adult doses, as shown in Figure 3.

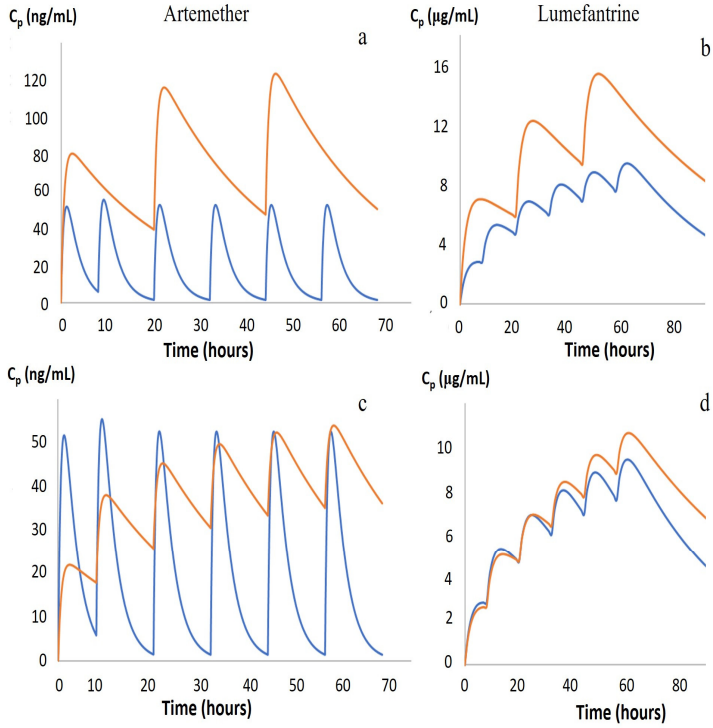

Figure 3. Plasma concentration time profiles of artemether and lumefantrine in adults and children. In (**a**,**b**), the dose-regimen has been changed for children in such a way that children take their medicine once a day, not at times 0, 8 h, then every 12 h for 3 days, where the pharmacokinetics for artemether (**a**) and lumefantrine (**b**) are shown. In (**c**,**d**), the dose of artemether has been adjusted for children to 0.34 mg/kg instead of 5–24 mg/kg (**c**); the dose for lumefantrine has been adjusted to 6 mg/kg instead of 29–244 mg/kg (**d**).

4. Discussion

4.1. Metabolism and CYP Enzyme Maturation

During development from fetus to adulthood, the liver undergoes development and metabolic enzymes take time to reach maturity. The size of the liver in infants and small children is relatively larger than that in adults. This may be reflected by variations in the activity of metabolizing enzymes with respect to liver size. Having a relatively larger organ will require relatively more blood flow, which will affect the disposition and can lead to either underexposure and treatment failure or overexposure with accompanying toxicity [39]. Cytochrome P450 (CYP) is a super-family of enzymes, categorized into various groups and subgroups based upon the similarity in the amino acid sequence. CYP3A, CYP2D6, and CYP2C9 together are responsible for almost 85% of the entire drug oxidation activities in people.

In embryonic life until late infancy, CYP3A7 is one of the important enzymes [61] when it comes to metabolizing drugs. Among the cytochrome P450 (CYP) enzymes, CYP3A predominates in the liver of adults and is responsible for the metabolism of almost 50–60%

of the entire marketed drugs at present. The expression of CYP3A4 slowly increases during childhood until it surpasses that of adults and then slowly decreases to the level of adults at the end of puberty. The levels of this enzyme rise to 50% of the value found in grown-ups between 6 and 12 months of age, affecting the pharmacokinetics of these drugs [38,39,61,62]. Therefore, changes in drug clearance caused by these enzymes may require adjustment in doses and/or dosing intervals in small children.

When it comes to the metabolism of artemether, CYP3A4 and CYP2B6 are responsible for immediate hepatic metabolism [38,63,64]. Lumefantrine, however, is absorbed more slowly and is largely metabolized by CYP3A4 to its active metabolite [63,64].

4.2. Dosage Calculation

Children from 2 months to 5 years are the age group that is most at risk of being hit by severe or cerebral malaria. Malnourished children are a population group of special concern, since absorption from the GI tract may take much longer time than in normal children due to intestinal villous atrophy in the jejunal mucosa, as previously discussed. Due to poor absorption in malnourished children, these children may require higher dosages, such as those recommended by the WHO, to ensure that some of the drug is taken up by the GI tract. Therefore, lumefantrine has been shown to have bioavailability as low as 5% in such children.

In general, there is little information available for malnourished children when it comes to pharmacokinetic parameters, although it is known that they are highly affected by nutritional status. Thus, it is currently necessary to use the same dosage estimation as for non-malnourished children. In general, the calculated doses of artemether and lumefantrine based on body weight should be lower (i.e., 0.34 mg/kg for AR and 6 mg/kg for LF) than the doses recommended by the WHO for non-malnourished children. An exception is malnourished children, in whom researchers have reported significantly decreased absorption compared to that in healthy children.

4.3. Pharmacokinetic Method

When comparing a once-a-day dosage in children (Figure 3a,b), which is not recommended by the guidelines, to the recommended dosage (Figure 2e,f), the graphs follow the adult PK better. In malnourished children, protein manufacturing is significantly reduced, resulting in low serum albumin and low lipoprotein concentrations that will affect protein binding, especially for lumefantrine, which is 99.7% bound to circulating proteins. However, because of the nature of α1-glycoprotein, levels of this protein fluctuate based on health conditions, e.g., levels may increase in the gut due to inflammation resulting from the pathophysiological condition in the gut mucosa of malnourished children.

There are multiple other factors that influence the pharmacokinetics of artemether and lumefantrine in malnourished children, such as the effect of food, that have dramatic effects on the absorption of both drugs. The reason is that food increases the secretion of bile salts that will improve the absorption of these lipophilic drugs. The bioavailability of lumefantrine in fasted or malnourished children may be as low as 4.7% [65]. Other influencing factors in malnourished children are the villous atrophy as well as the reduced bile salt concentration secreted into the duodenum. These factors will be important in understanding what happens when infected with malaria and how the infection will change the health status and alter the pharmacokinetics of both drugs.

It has been described that malaria may increase the half-life of lumefantrine as in healthy individuals the terminal half-life is 2 to 3 days while it is 4 to 6 days in falciparum malaria patients. Therefore, this may affect the steady-state plasma concentration. These factors remain to be studied. Various methods are used to scale the dosage from adults to children. Accurate and effective dosing of patients depend on an accurate estimation of the PK and PD of the drug. In turn, the PK and PD of a drug are dependent on the patient's physiological characteristics, which are unique for each patient as well as patient population.

Here, the model used was based on a one-compartment model, whereas real data showed that both drugs, artemether and lumefantrine, followed a two-compartment model, as seen in Figure 2a,b. However, pharmacokinetic values for artemether and lumefantrine in children are scarce, and some of the values have significant effects on the dose selected, bioavailability, volume of distribution, and elimination half-life. In addition, there is little information available for malnourished children when it comes to pharmacokinetic parameters, although it is known that these parameters are highly affected by nutritional status. This makes it difficult to estimate the accurate dose to be used in the development of a new formulation for artemether and lumefantrine for children.

4.4. Physiological Information

Physiological changes happen during the growth of a child. Body composition affects various parameters, such as the volume of distribution (V_d), the maturation of the liver and its enzymes, and how this affects the clearance and half-life of the drug as well as the plasma protein levels for different proteins that bind to the compound of interest. Lerman et al. [66] observed a 50% reduction in the concentration of AAG in the serum of preterm neonates compared to that of full-term neonates, while it was 300% higher in infants than in full-term neonates. However, from infants to adolescents, the AAG concentration only rose by 25%.

This indicates that the free fraction of drugs that binds to AAG may be highest in neonates and decline with an increase in age. Therefore, a reduction in plasma protein binding is not only because of a decrease in the overall amount (quantity) of plasma proteins, but there may also be a reduction in the binding affinity (quality) that affects the overall binding ability [15], resulting in altered pharmacokinetics and pharmacodynamics of drugs compared to adults. Having a high free drug concentration may, however, have its drawbacks, such as altered volume of distribution, clearance, and half-life, since free drug is cleared more easily than drug bound to plasma proteins and red blood cells. In severe cases of malaria, such as cerebral malaria, drug distribution to the brain is required.

Lipid-soluble drugs have more easy access across the blood-brain barrier (BBB), and this access is even easier in very young children compared to older ones and adults. This is because the BBB has not matured enough to prevent drugs from penetrating across and into the brain [67]. The manufacturing of these plasma proteins is highly affected by the nutritional status of the individual, so children that are malnourished may have a decreased concentration of these proteins [68]. Human serum albumin (HSA) is the most abundant protein in plasma and binds to most drugs, followed by α_1-acid glycoprotein [66,68]. The normal plasma concentrations of both proteins are lower in neonates and infants than in older children [66], so the effect of malnutrition is more dramatic in young children. Therefore, an increase in the free fraction of the drug may be expected in children with accompanying consequences.

This information can be used to estimate the dose. However, there are not many pharmacokinetic parameters available for children, making a real extrapolation difficult. For lumefantrine, the published V_d is very high for children and the bioavailability is very low, especially for children that take the medicine in a fasted state. These factors have a dramatic effect on the pharmacokinetics if they are true. If the V_d is as high as described here, the dose needs to be significantly increased to obtain a similar plasma concentration as in adults. Some authors have claimed that patient age and gender have no effect on any of the main pharmacokinetic parameters, such as absorption rate constant (k_{abs}) and Cl [69].

According to Ashley et al. (2007), the absorption of lumefantrine may increase 6-fold when taken with soy milk. In the case of artemisinin combination therapies (ACTs), poor absorption of lipids and fats due to villous atrophy can affect the absorption of lipid-soluble ACTs [70]. Decreased exposure to lumefantrine has been reported in children with severe acute malnutrition [47,71,72]. In addition, the metabolism of antimalarial drugs can be decreased due to low plasma albumin and other plasma proteins concentrations, as previously explained [72]. Hypoalbuminemia is more acute in kwashiorkor than in

marasmus [47,71,72]. Hepatic metabolism of antimalarial drugs can be dysregulated and decreased or increased depending on whether the malnutrition is predominantly a protein deficiency or global malnutrition [73]. Therefore, a deeper understanding of different types of malnutrition and their effects on pharmacokinetics is important so that pediatric doses can be adjusted accordingly [47,71,72].

The monitoring of hepatic-metabolized antimalarials is, hence, crucial in children with malnutrition. Interstitial water retention is another critical pathophysiological change in SAM that also affects the pharmacokinetics of antimalarial drugs [47]. Water retention increases the volume of distribution of drugs and, in turn, can cause suboptimal drug exposure of a standard dose [47,72]. Children with undernutrition risk dosing inaccuracies such as underdosing for weight when the dosing is only based on age, or overdosing for age when the dosing is based on weight alone [40]. Dose optimization for ACTs, for the recommended treatment for uncomplicated *P. falciparum* malaria in most malaria-endemic countries, is crucial for optimum dosing efficacy, safety, and avoiding parasite resistance [74].

A recent meta-analysis showed a higher risk of treatment failure in malnourished children than in well-nourished children in Africa [75]. Furthermore, during treatment with AR-LF for uncomplicated malaria, younger children under 3 years, including underweight-for-age children, had a 23% lower concentration of the drug adjusted for mg/kg at day 7 in comparison with well-nourished children of the same age [48]. To optimize the dose for malnourished children, pharmacokinetic and pharmacodynamic data are essential. In tackling the question of the effect of SAM on the pharmacokinetics and pharmacodynamics of artemether-lumefantrine for the treatment of uncomplicated malaria in children, a study in 2019 showed essential findings [76]. The study found that all malnutrition was associated with reduced absorption of lumefantrine [76]. Specifically, the mid-upper-arm circumference (MUAC) was the most significant covariate, indicated by a 25.4% decreased absorption per 1 cm reduction in MUAC [76]. In addition, the study depicted that the lower exposure of lumefantrine resulted in an increased risk of acquiring a new *Plasmodium falciparum* infection during the follow-up period [76]. Such results emphasize the essential need for dose optimization for ACTs in children with SAM to control for the poor absorption and achieve the intended efficacy in these children at risk of the adverse effects of malaria and malaria re-infection.

5. Conclusions

Individual variations in patients in a defined patient population affect the PK and PD of drugs. Children under the age of 5 years are not small adults, and their physiology, liver enzyme maturation, and serum proteins have not fully developed and may, therefore, significantly influence the pharmacokinetics of drugs and how they behave in the body. Obese children have a greater quantity of body fat, which may lead to an increased volume of distribution of lipid-soluble drugs [77]. Therefore, when designing a dose regimen, it is important to know and include the distinctions in physiology at various phases of development that influence the pharmacokinetics of various drugs when estimating the dose in young children. The possibility of estimating the accurate dose in children depends on the availability of more pharmacokinetic data derived from clinical studies.

Malnutrition, lack of clean water, and infections (e.g., helminthics) may affect the pharmacokinetics of drugs in children when they need to receive acute treatment, such as for malaria. It is, therefore, important to understand how these factors affect how the drug is handled by children living in parts of the world where severe and cerebral malaria are endemic. Dose adjustments in children that are categorized as having normal weight, compared with obese or malnourished children, need to be based on the pharmacokinetic changes that happen in these situations. Therefore, accurate and effective dosing of patients depend on an accurate estimation of the pharmacokinetics (PK) and pharmacodynamics (PD) of the drug.

Due to the lack of real pharmacokinetic data for children in the literature, a clinical study needs to be conducted to verify whether the suggested doses can be clinically efficacious.

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Paper III

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BIOEQUIVALENCE STUDY OF LUMEFANTRINE RECTAL ENEMA AND THE COMMERCIALLY AVAILABLE GENERIC ORAL SUSPENSION. A PILOT STUDY

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ABSTRACT: Children under five years of age, with severe or cerebral malaria, cannot consume oral medication especially if they are vomiting or are unconscious. In such situations they are given an injectable drug until they can tolerate oral medication. The situation is bad in Sub-Saharan Africa, especially in rural areas as children are sometimes referred to the closest referral health care facility for proper management. The aim of this study was therefore to conduct a pilot study to estimate the bioavailability of lumefantrine (LF) when administered as a rectal enema, and compare it with a commercially available oral suspension, in rabbits. The study was conducted on six healthy rabbits in an open randomized, crossover three sequence, single dose study, where each rabbit received rectal and oral formulations. The oral formulation was administered under fed and fasted conditions. A two-week washout period was allowed between the experiments. LF was quantified in rabbit plasma using ultraperformance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). Results showed that the relative bioavailability of rectal LF was about four times higher than oral. The observed data suggest that a significant adjustment in the dose will be required when LF is administered via the rectal route. The data provide important information for the next step in finding a method to provide a rescue treatment for children with severe or cerebral malaria.

INTRODUCTION: Malaria is a global burden with high mortality reported in children in Sub-Saharan Africa [1-3]. In severe malaria, children can become anemic, have decreased levels of glucose, or cerebral malaria, just to mention a few.

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The susceptibility to other infections increases in these children and they can even develop recurring malaria [4, 5]. It is reported that about US$ 3.1 billion was spent globally due to malaria in 2018 [3, 6].

The World Health Organization (WHO) recommends the use of an artemisinin-based combination therapy (ACTs) for treatment of Plasmodium falciparum malaria. Artemether (AR) and lumefantrine (LF) combination product is one of the ACTs that has been approved for treating uncomplicated *P. falciparum* malaria [7, 8]. Due to

the development of drug resistant malaria parasite, a combination product is the best way to fight such a risk.

AR is derived from artemisinin and works by inhibiting synthesis of proteins in the parasites through production of free radicals [9, 10]. AR has fast onset of action and a short half-life of about (2-3 hours) [9]. It is available as an oral fixed dose combination with LF or as monotherapy dissolved in oil, that is given intramuscularly (IM).

LF belongs to the class of antimalarials called arylamine alcohol [11]. It kills parasites by destroying the hemoglobin in the parasites [9, 10]. Absorption of LF is highly dependent on intake of a fatty meal and may have a lag time of up to 2 hours [9, 12]. LF acts slowly, highest plasma concentration is obtained after 6-8 hours; and has a long elimination half-life of about 2-3 days in healthy human subjects and 4-6 days in *P. falciparum* infected patients [13]. Both AR and LF are highly protein bound i.e., 95.4 % and 99.7 % respectively [11, 13].

Rectal administration of artemisinin derivatives has been reported to achieve acceptable therapeutic outcomes including in severe disease [14]. Currently products available to treat severe falciparum or cerebral malaria contain one drug only in the form of injectables. Children with severe falciparum malaria are given injections of either artesunate, AR or quinine depending on which one of the three monotherapy injectables is available until they can tolerate oral ACTs. When using injections, AR IM is preferred to quinine [15]. In absence of these injectables, especially at the primary level of health care, children below 6 years of age are given rectal artesunate and referred to the next level of health care [14].

However, rectal artesunate is a monotherapy. Due to an increase in emergence of drug resistant malaria parasites, there is a need for a rescue combination product. Although combination products for treating malaria are available, they cannot be used in unconscious and vomiting children. Suppositories for combination antimalarial products have been marketed but are not suitable in the hot temperatures of some Sub-Saharan countries. In addition, the limited volume of fluid in the rectum makes it a challenge as the suppository must either melt or dissolve depending on the nature of the suppository base before the drugs are released into the rectal fluid. Therefore, if a liquid rectal drug delivery system containing ACTs could be made available, that formulation could be an alternative in these children.

Drugs administered rectally can partially bypass metabolism in the liver when the dosage form is inserted on the lower part of the rectum [16]. There have been challenges in solubilizing lumefantrine due to its very poor solubility. A combination dosage form such as a rectal enema, containing both drugs, to be used as an emergency rescue treatment of children that have malaria was recently developed. The aim of this study was therefore to conduct a pilot study to investigate the absorption of lumefantrine when administered as a rectal oily solution, and compare it with a commercially available oral suspension, in rabbits.

When evaluating the bioavailability of a new formulation the reference product is an IV formulation. In certain situations, however, relative, or comparative bioavailability is used, such as when the drug is insoluble and cannot be administered as an IV. In such situations both the reference and test formulations are extravascular products. LF is such a drug as it is not available as an injectable formulation. Lumefantrine is highly dependent on food intake, probably due to the augmentation of the bile salt production, since it has been shown that the relative bioavailability is found to be increased by 16-fold when administered with a high fat diet [12, 17].

METHODS:

Test Materials: Lonart® 20/120 (Bliss GVS Pharma Ltd, India) powder for oral suspension was kindly provided by a local community pharmacy in Malawi. The rectal enema was prepared at the Faculty of Pharmaceutical Sciences at the University of Iceland and contained 8.7 mg artemether and 52 mg lumefantrine per mL. The oral powder was reconstituted with water to make a suspension before administration while the rectal enema was a preformulated formulation that contained AR-LF dissolved in a lipophilic solvent. Both drugs were kindly provided by Mangalam Drugs and Organics Limited (Mumbai, India).

Animals: The study was conducted at the Department of Pharmacology, Faculty of Veterinary Medicine at the University of Murcia, Spain, between the months of May – July 2022. It was approved by the Bioethical Committee of the University of Murcia, Spain (approved experimental protocol number (os 811/2022) and was carried out according to the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals. All efforts were undertaken according to the "3R principles" to reduce the number of animals used in the study and optimize experimental protocols for obtaining maximum data from each tested animal. A limited number of rabbits were used since this was a pilot study to investigate LF absorption from the rectal solution.

New Zealand White (NZW) male and female rabbits were purchased from Granja San Bernardo (Spain). The materials used for the animal experiments consisted of 1.8 mL Eppendorf tubes, amber colored glass vials (2 mL), 22G catheters, 2.5 mL syringes, normal saline (0.9 % saline), alcohol 96 %, were purchased from Proquilab (Murcia, Spain), heparin 1000 IU/mL and female catheters were purchased from Aleman Pharmacy (Murcia, Spain) weighing scales were purchased from Mettler Toledo (Barcelona, Spain), Vortex and mini spin centrifuge were purchased from Merck Life Science (Madrid, Spain).

Study design: A total of six healthy NZW rabbits weighing between 3.75 to 5.14 kg and aged 1-2 years old were housed in individual cages with free access to food and water *at libitum* and maintained on a 12/12 h light /dark cycle at controlled and fixed temperature and humidity at the laboratory animal care facility at the Faculty of Veterinary Medicine at the University of Murcia, Spain. Before the experiments, all animals were allowed to acclimatize for several days.

The study was an open randomized, three sequence single dose crossover study. The animals were weighed and randomized into three groups. In each experiment animals were randomized to receive either: (a) the oral suspension under fasted state; (b) or oral formulation under fed state; (c) or the rectal formulation. The animals that received the oral formulation under fasted state were starved 12 hours before the study but had free access to water. There was a two-week washout period between each experimental sequence. Each rabbit was dosed with the corresponding volume of either the oral suspension or rectal enema equivalent to 24 mg/kg of LF per administration. All the animals were observed daily for 14 days, after the dosing, for any possible adverse events.

Blood Sampling: A 22G catheter attached to a 2.5 mL syringe was used to collect blood samples (about 1.2 mL) from the marginal ear vein of each rabbit before and after dose administration at the following time intervals: 0, 30, 45, 60 and 90 min, then after 2, 4, 6, 8, 10, 24, 48, and 96 hours**.** Considering the 13 samples that were taken, the total volume did not exceed 10% of the total maximum volume of blood that could be collected without significant changes in apparent volume of distribution and was therefore adequate.Syringes (2.5 mL) were flushed/rinsed with approximately 0.2 mL heparin (1000 IU/ mL) and used to collect blood samples into appropriately labelled 1.8 mL transparent Eppendorf tubes.

Blood samples were immediately centrifuged on a mini spin Eppendorf at 12,000 rpm for 10 min. Plasma was collected and divided into two amber colored glass vials (2 mL) and put in a freezer at -80 ± 3°C. At the end of the study, samples were packed and shipped to Iceland for analysis, using *reusable iceless containers* provided by World Courier® shipping company. Upon arrival in Iceland the samples were stored in a freezer at -80 ± 3°C until analysis.

Preparation of Standard Solutions: Stock solution of LF was prepared in MeOH with 0.5 % formic acid. The internal standard (IS), Lumefantrine-d9 was dissolved in MeOH with 0.5% formic acid. All stock solutions were stored at -20°C until use. Working solutions containing LF were prepared using serial dilution with MeOH: water (50:50 v/v).

Eight calibration samples and three quality control (QC) samples were prepared by spiking blank human plasma with 10 µL of appropriate working solution to 90 µL of blank plasma to create standards and quality control samples. The concentration of the calibration samples in plasma were 5, 10, 20, 50, 250, 500, 2000 and 5000 ng/ml

for LF. QC samples in plasm were 15, 150 and 3000 ng/mL.

Plasma Sample Preparation: Rabbit plasma sample (100µL) was added to a 96 well plate followed by 300 µL of MeOH + 0.5% acetic acid containing IS, plate was sealed with cap mat and samples vortexed for 1 min at 1050 RPM. Samples were then centrifuged at 2500 g for 10 min. The supernatant (200 µL) was transferred into a new 96 well plate. The sample (2 µL) was injected into the UPLC-MS/MS system. The limit of quantification (LOQ) was 5 ng/mL.

UPLC_MS/MS Method for Quantification of Lumefantrine: The quantification of LF was performed on a Waters Acquity I-Class UPLC system, coupled to a Waters Xevo TQ-XS triple quadrupole mass spectrometer equipped with electrospray ionization (ESI) probe. Nitrogen was used as a desolvation and cone gas and high purity argon as a collision gas. Source temperature was set to 150°C and desolvation gas temperature was 600°C,at a flow rate of 1000 L h^{-1}; cone gas flow rate 150 L h^{-1}.

The analytical column, Acquity Premier BEH (1.7 µm, 50 × 2.1 mm) (Waters Corporation, Wexford, Ireland), was maintained at 30 °C. The injection volume was 2.0 µL and the sample manager temperature was maintained at ambient temperature. The gradient system consisted of mobile phase A: 0.5% formic acid in water and mobile phase B: 0.5% formic acid in methanol, at a flow rate of 0.50 mL/min.

Initial conditions starting at 32% mobile phase A followed by a linear gradient to 88% of mobile phase B at 2.0 min then back to initial conditions at 2.1 min and then held for 1.9 min. The total chromatographic run time was 4.0 min and the retention time of lumefantrine was 1.6 min. The mass spectrometer was optimized for analyzing LF and lumefantrine-d9 using multiple-reactions monitoring in the positive ESI mode to monitor precursor ion → product ion m/z 527.95 → 509.92 for lumefantrine and 538.90 → 521.03 for lumefantrine-d9. Lumefantrine-d9 was used as an internal standard for LF.

Pharmacokinetic Calculations and Statistical Analysis: Non compartmental analysis (NCA) was used to determine the PK profile of LF using PK Solver 2.0, an Add–in software for Microsoft Excel and Pkanalix 2021R2 (Lixoft SAS, a Simulations Plus Company). Microsoft® Excel® for Microsoft 365 was used for calculations. QI Macros 2023 an Add-in program for Microsoft Excel was used for statistical analysis. Nonparametric tests i.e., Kruskal-Wallis test was used to determine whether a significant difference among the median of three groups existed or not ($\alpha = 0.05$).

The Mann Whitney test was used to compare the medians of two groups using the Bonferroni correction to decrease the probability of committing a type 1 error. The Holm – Bonferroni method was used to decrease the probability of committing a type 2 error since the single step Bonferroni lack statistical power. A t-test was also used to detect the presence or absence of a significant difference between the means of two groups.

The $C_{max}$and t_{max}were obtained directly from the plasma concentration time data by PK Solver and Pkanalix software. The area under the plasma time curve (AUC) from time zero to the last observed time was calculated using the linear trapezoidal method by the Software. The AUC, extrapolated from the last data point to infinity, was estimated by dividing the concentration at the last time point by the terminal elimination rate constant using PK Solver and PKanalix.

Equation 1 was used to calculate relative bioavailability.

$$F = AUC_{test}\ (0\text{-}96\ h) \times D_{reference} / AUC_{oral}\ (0\text{-}96\ h \times D_{test} \quad \text{..(Eq 1)}$$

Where F is the bioavailability, AUC0-96h is the area under the curve for the test formulation (rectal or oral fasted), and the reference formulation (oral fed); and D is the dose for same formulations. Total body clearance was calculated using Equation 2 and apparent plasma clearance using Equation 3:

$$Cl = k_e \times V_d \quad \text{..... (Eq 2)}$$

$$Cl/F = D / AUC_{0\text{-}\infty} \quad \text{..... (Eq 3)}$$

Where Cl is the clearance and Cl/F is the apparent plasma clearance of drug after extravascular administration. The volume of distribution (Vd)

and the apparent volumeof distribution (Vd/F) was calculated using Equation 4 and 5:

$$V_d = D/C_{max} \quad \text{(Eq 4)}$$

$$V_d/F = Cl/F/k_e \quad \text{(Eq 5)}$$

Where Cmax is the maximal plasma concentration and ke is the elimination rate constant. AUC from last observed data to infinity was calculated using Equation 6:

$$AUC_{96-\infty} = C_{last} / k_e \quad \text{(Eq 6)}$$

Where Clast represents the last observed concentration, after 96 hours.

RESULTS: Both the reference and test formulations seemed to be well tolerated in the animals, as no signs indicated discomfort or irritation. Six rabbits participated in the study. Leakage of the rectal formulation occurred in a female rabbit a few minutes after administration and, therefore, it was decided to repeat the rectal formulation and skip the oral fasted sequence. The relative bioavailability (F) of LF calculated from time zero to 96h time point following rectal administration with respect to the oral administration dosed under fed state was 4.18 (418%) as shown in **Table 1**. The Table shows the pharmacokinetic parameters following oral and rectal administration of LF. The relative bioavailability of the rectal enema with respect to the oral administration dosed under fasted state was 4.50 (450%). The maximal plasma concentration following rectal administration was found to be 1,924 ng/mL, compared to 883 ng/mL following oral (fed) animals.

The elimination rate constant, however, was comparable for all animals. The t_{max} was long for oral administration as expected or about 6 -10 hours. However, for the rectal group the t_{max} was found to be unusually long, or about 14.6 hours. For LF, Kruskal-Wallis test showed that a significant difference ($\alpha < 0.05$) existed among treatment groups, both with respect to C_{max} and AUC. Post hoc analysis using the adjusted Holm-Bonferroni α-value revealed that the difference was only significant between the oral groups and the rectal group, but no significant difference was found between the two oral groups.

TABLE 1: THE PHARMACOKINETIC PARAMETERS OF LUMEFANTRINE, FOLLOWING ORAL (FASTED OR FED) ADMINISTRATION OF THE PRODUCT LONART® 20/120 SUSPENSION, AND COMPARED WITH RECTAL ADMINISTRATION OF LF. ALL ANIMALS RECEIVED 24 MG LF PER KG BODY WEIGHT. SIGNIFICANCE WAS MEASURED BETWEEN ORAL (FED) ANIMALS AND RECTAL ADMINISTRATION

	Rectal	Oral (fed)	Oral (fasted)	*p*-value [1]
D (mg/kg)	**24**	**24**	**24**	
C_{max} (ng/mL)	1,924 ± 658	883 ± 471	333 ± 301	0.012
t_{max} (h)	14.6 ± 7.2	6.0 ± 1.3	10.0 ± 8.1	0.077
V_d/F (L/kg)	14 ± 4	45 ± 48	82 ± 73	0.167
V_d (L)	272 ± 204	198 ± 195	359 ± 243	0.537
k_e (h^{-1})	0.030 ± 0.017	0.037 ± 0.014	0.044 ± 0.020	0.465
$t_{½}$ (h)	33.8 ± 25.1	24.3 ± 18.9	18.4 ± 7.7	0.475
MRT (h)	48.0 ± 31.8	31.1 ± 21.0	33.2 ± 6.5	0.306
Cl/F (L/h/kg)	0.36 ± 0.15	1.6 ± 1.9	4.6 ± 2.9	0.162
Cl(L/h/kg)	1.4 ± 1.1	1.6 ± 1.9	3.0 ± 1.7	0.840
AUC_{0-96h} (ng/mL·h)	53,501 ± 26,404	16,815 ± 10,426	11,576 ± 9,986	0.008
AUC_{0-inf} (ng/mL·h)	63,861 ± 37,445	17,575 ± 10,186	11,893 ± 10,087	0.027
F_{rel}	4.18 ± 2.73	(Reference value)	0.58 ± 0.282[2]	

1 = comparing oral (fed) v.s. rectal administration; 2 = (n = 4) because an outlier was removed

When the median peak times were analyzed instead of average, the t_{max} was found to be 6 h (4–8 h), 6 h (4–24 h) and 10 h (10-24 h) following oral fed, oral fasted and rectal administration, respectively. No significant difference was found for the half-lives of LF. Similarly, the median weight adjusted apparent clearance (*Cl/F*) was found to be 0.85 L/h/kg, 2.33 L/h/kg and 1.12 L/h/kg following oral fed, oral fasted, and rectal administration respectively. The median weight adjusted apparent volume of distribution (V_d/F) was found to be 27 L/kg, 71 L/kg, and 47 L/kg, for oral fed, oral fasted and rectal administration, respectively. The median values for the mean residence time (MRT) for oral fed, oral fasted and rectal administration were 25h, 31h, and 36h respectively.

Fig. 1 shows the average plasma concentration time curve following oral fed, oral fasted and rectal administration to rabbits. The amount of LF absorbed following rectal administration of LF in a lipophilic vehicle was found to increase the amount absorbed over 4-5-fold (up to 9.4-fold) compared to oral administration.

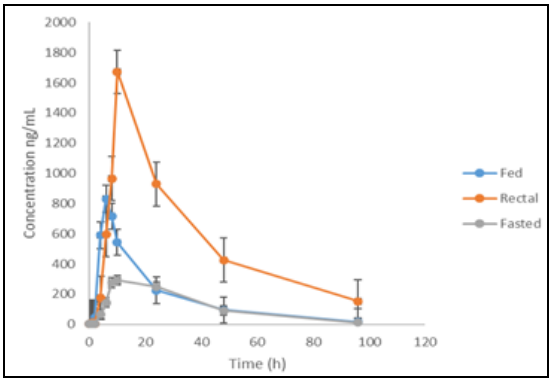

FIG. 1: MEAN PLASMA CONCENTRATION TIME PROFILE FOR LUMEFANTRINE FOLLOWING ADMINISTRATION OF LONART® 20/120 TO HEALTHY RABBITS. BLUE, ORAL DELIVERY TO FED RABBITS; GRAY, ORAL DELIVERY TO FASTED RABBITS; AND ORANGE, RECTAL ADMINISTRATION OF THE ENEMA. ERROR BARS REPRESENT STANDARD ERROR

DISCUSSION: Findings from this pilot study indicate that the relative bioavailability of LF following rectal administration was at least four times higher than following oral suspension. When compared to fasted animals, the bioavailability was found to be even higher or ≥6 fold indicating that rectal administration was superior to oral delivery, and that access to food seems to be important for oral administration, since the bioavailability drops down to 0.58 (when one outlier is eliminated) in animals that were not allowed to eat 12 h prior to the study.

If these data reflect a real clinical situation, a 120 mg dose of LF could be reduced to about 30 mg. If the pediatric dose of 120 mg is calculated based on the fact that children that cannot be fed, should get enough drug, then it should be possible to reduce the dose down to about 17 mg [18]. To follow up on this work, it will be necessary to recalculate the clinical dose required [18]. Although rectally administered drugs partially bypass the first pass metabolism, this does not explain the difference observed in the bioavailability. Especially when other pharmacokinetic parameters are studied, such as t_{max}. Orally administered LF requires bile salts to become solubilized, and absorbed, this explains the difference between oral fasted and oral fed bioavailability data. If bile salts are limited, for example when a child does not have access to food, or if the child is unconscious or cannot take food orally, the bioavailability will be lower or even negligible. Formulations, especially bio adhesive formulation, that can keep LF in a soluble form throughout the study may explain current findings.

Clinical data have shown even more differences between fasted and fed individuals receiving LF [12, 13] although our data does not show a significant difference. One possible reason could be that 2 h after the study started, the rabbits gained free access to food and water, which may have facilitated secretion of bile salts. Something that would not be the case in disease management situations.

Data in **Table 1** indicates large individual differences which is in line with data reported in literature. Considerable inter individual variability has been reported when LF is administered orally in clinical studies done in human beings[13]. Absorption of drugs following rectal administration is also reported to be erratic and highly variable [16, 19] but still beneficial when they produce desired pharmacokinetic profiles and therefore can be used in situations where patients cannot swallow oral medication [19, 20]. In this pilot study, good information was gathered, and the next step would be to carry out a full-size bioequivalence study for proper statistical analysis, as well as metabolite analysis.

Following oral absorption, a lag time is reported for LF solid dosage forms [21] as the drug must first disintegrate and become dissolved before being absorbed. The observed time to peak plasma concentration (t_{max}) following oral administration in rabbits was similar to that observed in human beings (6–8 h) [22, 23]. Other researchers have also reported peak times of 6h and 8h, respectively, in a bioequivalence study of two generic AR–LF products dosed orally in rabbits [24]. However, the case is different with rectal enema since the formulation tested contained dissolved LF in a

lipophilic vehicle. Therefore, the drug bypassed the disintegration and dissolution stages, and the absorption was expected to be rapid. The observed median peak time following rectal administration, however, was found to be 10 h, something that is quite long. This long peak time (t_{max}) may partly be explained by the formulation components/ excipients and the anatomical difference between rabbit rectal anatomy, compared to human rectal anatomy as shown in **Fig. 2.**

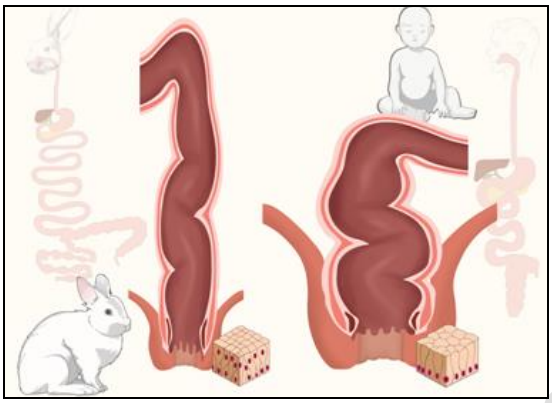

FIG. 2: SHOWING THE ANATOMICAL DIFFERENCES BETWEEN THE RABBIT AND HUMAN RECTUM, INCLUDING THE CELLULAR STRUCTURE INSIDE THE RECTAL MUCOSA

The thickness of the rectal mucosa of rabbits weighing 2 kg was reported to be 280 µm [25] while in humans it is 150 µm [26]. Therefore, the physiology of the rabbit rectal mucosa may be more resistant to allowing compounds such as LF to be transported into the systemic circulation. In addition, human rectal mucosa does not have much water, and the transport rate and transport capacity of LF across the rectal mucosa is not known. Therefore, the long t_{max} observed in rabbits require additional studies, where the difference of human and rabbit rectal anatomy and physiology will be examined [27].

Having a bio adhesive lipophilic formulation may allow the formulation to stick to the rectal mucosa, transporting the drug slowly across the rectal mucosa. This needs to be studied in more detail when human trials will be planned, or in more anatomically similar animal species. Drugs that are absorbed from the rectum may be taken up through three veins: the superior, middle, and inferior hemorrhoidal (rectal) veins. The superior hemorrhoidal vein is connected to the portal venous system, so drugs absorbed into this vein will undergo first pass hepatic metabolism before reaching the systemic circulation. The middle and inferior rectal veins absorb the drug(s) directly into the systemic circulation bypassing the first pass hepatic metabolism [19]. In addition, lymphatic drainage may also happen through the rectal mucosa, which will bypass first pass metabolism, and may end up in the systemic circulation, something that may happen for very lipophilic drugs [16].

A significant difference was found when comparing the C_{max} between the rectal and oral administration groups, showing about 2.2-fold higher C_{max} for the rectal formulation compared to oral (fed) group and 5.8 times higher than in the oral fasted group. Based on this data, it may be required to recalculate the rectal dose, and administration schedule for a possible AR-LF rectal enema.

The mean apparent clearances of LF were found to be around 1.7 L/h, 7 L/h and 21 L/h following rectal, oral fed and fasted states respectively. The clearance will be important to determine the maintenance dose and frequency of drug administration[28]. Because of the long t_{max}, the distribution phase could not be studied and the concentration in different organs was not part of this study but could be an interesting future study[29, 30].

No significant difference was found for the half-lives of LF, indicating similar metabolisms for oral and rectal routes. The median half-life ($t_{½}$) for LF in this study was found to be about 17 h and the average half-life of 24 h when dosed orally under fed state, while in healthy human individuals the reported half-life is from 48-72h. The metabolic rate in rabbits may be faster than in humans, giving a shorter half-life. The median and average half-lives following rectal administration were 22 h and 34 h respectively.

Oral absorption of LF is affected by multiple factors, that will affect the pharmacokinetics of this drug, and need to be considered. It has been reported that LF absorption may rise by 5-fold, when taken with soy milk and16 fold when taken

with a fatty meal [12], since bile salts are important to ensure complete solubilization. A formulation that does not require fatty food intake prior to drug administration, is, therefore, very important when treating critically ill patients with severe or cerebral malaria. Therefore, rectal enema could be useful in such situations.

CONCLUSIONS: AR-LF oral suspension and rectal enema were successfully administered to healthy rabbits and compared. The bioavailability of LF showed that a significantly higher bioavailability was found following rectal administration, compared with the oral route. The t_{max}, however, was surprisingly long and required some additional studies, especially with respect to the anatomical and physiological difference between rabbits and humans.

The observed data suggest that a significant adjustment in the dose will be required when LF is administered via the rectal route, to receive comparable plasma levels as for healthy adults. Although this pilot study had low power, due to the small sample size, the data provide important information for the next step in finding a method to provide a rescue treatment for children with severe or cerebral malaria. A clinical study with high power should be conducted.

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Institutional Review Board Statement: Not applicable.

Informed Consent Statement: The study was approved by the Bioethical Committee of the University of Murcia, Spain (approved experimental protocol number os 811/2022).

Patents: E.K.G.M, B.R.S and S.G. filed a patent application back in 2019 on the dissolution of lumefantrine and artemether.

Data Availability Statement: N/A

CONFLICTS OF INTEREST: The authors declare no conflict of interest.

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Reviewer's recommendations:

1. Specify designation and current full address of corresponding author.
2. Check for spelling, grammar and punctuation error(s).

Appendix A

The two level full factorial design with three quatitative factors can also be illustrated in table form as in Table A. The standard run order is represented by the column containing numbers up to eight; for instance, the first run is made at low setting of all three factors.

Table A. Illustration of a two level full factorial design with three quantitative factors set at fixed levels, low or high level. Adopted form Das and Dewanjee 2018.

Run/experiment	X_1	X_2	X_3
1	low	Low	low
2	high	Low	low
3	low	High	low
4	high	High	low
5	low	Low	high
6	high	Low	high
7	low	High	high
8	high	High	high

Appendix B

Gradient elution Based on the results after testing the two columns, four gradient elution methods were tested using the Kinetex® column only. The mobile phases consisted of 0.1 % formic acid in ACN and 0.1% formic acid in water and run at a flow rate of 1.3 mL/min. Stock solutions were diluted with acidic ACN and acidic water (50:50 % v/v) and 20 µL was injected at 25 °C at 218 nm, 236 nm and 254 nm. Tables 21, 22, 23, and 24 show the gradient elution methods that were tested. In Table 21 mobile phases A and B (MP A and MP B) consisted of acidic ACN and acidic water 50:50 and 95:5 % v/v respectively.

Table B. Gradient elution based on 0.1 % formic acid in ACN and 0.1 % formic acid in water where acidic ACN to acidic water for A was (50:50) and MP B (95:5) using the kinetex column

Time (min)	0.1% formic acid in can	0.1% formic acid in water	Curve
0.0	50	50	5 (linear)
0.0	50	50	5 (linear)
1.0	50	50	5 (linear)
1.5	95	5	5 (linear)
7.0	95	5	5 (linear)
8.0	50	50	5 (linear)
10.0	50	50	5 (linear)
10.0	Stop run		

Appendix C

Table C1. Gradient elution based on 0.1 % formic acidin ACN and 0.1 % formic acid in water where acidic ACN to acidic water for A was (72.5:27.5) and MP B (95.0:5.0)

Time (min)	0.1% formic acid in can	0.1% formic acid in water	**Curve**
0.0	72.5	27.5	5 (linear)
0.0	72.5:27.5	27.5	5 (linear)
1.0	72.5:27.5	27.5	5 (linear)
1.5	95:5	5	5 (linear)
5.0	95:5	5	5 (linear)
5.5	72.5	27.5	5 (linear)
7.0	72.5	27.5	5 (linear)
7.0	Stop run		

Table C. Gradient elution based on 0.1 % formic acid in ACN and 0.1 % formic acid in water where acidic ACN to acidic water for A was (95:5) and MP B (50:50)

Time (min)	0.1% formic acid in can	0.1% formic acid in water	Curve
0.0	95	5	5 (linear)
0.0	95	5	5 (linear)
1.0	95	5	5 (linear)
1.5	50	50	5 (linear)
5.0	50	50	5 (linear)
5.5	95	5	5 (linear)
7.0	95	5	5 (linear)
7.0	Stop run		

Appendix D

Table D. Gradient elution based on 0.1 % formic acid in ACN and 0.1 % formic acid in water where acidic ACN to acidic water for A was (50:50) and MP B (95:5)

Time (min)	0.1% formic acid in can	0.1% formic acid in water	**Curve**
0.0	50	50	5 (linear)
0.0	50.	50	5 (linear)
1.5	50	50	5 (linear)
2.0	95	5	5 (linear)
5.0	95	5	5 (linear)
5.5	50	50	5 (linear)
7.0	50	50	5 (linear)
7.0	Stop run		