The relationship between the insecticide dichloro-diphenyl-trichloroethane and chloroquine in *Plasmodium falciparum* resistance

by

Hazel Beverly Makowa

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Supervisor: Prof. Marina Rautenbach
Faculty of Science

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Declaration

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March 2012
Summary

Dichloro-diphenyl-trichloroethane (DDT) was extensively used in agriculture pest control and is still used for indoor residual spraying to control malaria. The lipophylicity of DDT and its breakdown product dichloro-diphenyl-dichloroethylene (DDE) dictates that they associate with membranes, lipids and hydrophobic proteins in the biological environment. Their poor degradable nature causes DDT and DDE to persist for decades in the environment and in individuals who are or were in contact with the pesticide. In many countries the synchronised resistance of the mosquito vector to insecticides and the malaria parasite towards antimalarial drugs led to a drastic rise in malaria cases and to malaria epidemics. This study assesses the influence of low level exposure of DDT and DDE on chloroquine (CQ) resistance of the dire human malaria parasite, *Plasmodium falciparum*.

The *in vitro* activity of *p,p'*-DDT and *p,p'*-DDE towards blood stages of chloroquine sensitive (CQS) *P. falciparum* D10 and chloroquine resistant (CQR) *P. falciparum* Dd2 was determined using two complementary *in vitro* assays (Malstat and SYBR Green 1). The 50% inhibition concentrations (IC50s) of *p,p'*-DDT and *p,p'*-DDE were found to be ±14 to 38 µM (5-12 µg/mL) and highly similar towards CQS and CQR *P. falciparum* strains. This result indicated that the proteins involved in CQ resistance have no effect on the activity of the insecticide DDT and its breakdown product DDE.

In order to assess the influence of DDT and DDE on CQ activity, *in vitro* fixed ratio drug combination assays were performed, as well as isobologram analysis. We found that CQ works in synergy with *p,p'*-DDT and *p,p'*-DDE against CQS *P. falciparum* D10. However, both *p,p'*-DDT and *p,p'*-DDE were antagonistic toward CQ activity in CQR *P. falciparum* Dd2. This indicated that *p,p'*-DDT and *p,p'*-DDE do have an effect on CQ resistance or on the action of CQ via a target other than hemozoin polymerization. The observation of reciprocal synergism of *p,p'*-DDT and *p,p'*-DDE with CQ against CQS D10 and antagonism against CQR Dd2 strain is highly significant and strongly indicates selection of CQ resistant strains in the presence of *p,p'*-DDT and *p,p'*-DDE. People who have low levels of circulating DDE and/or DDT could be at a high risk of contracting CQR malaria. However, medium term (nine days) DDE exposure of CQS *P. falciparum* D10 did not induce resistance, as no significant change in activity of CQ, *p,p'*-DDT and *p,p'*-DDE towards blood stages the CQS strain was observed. This exposure was, however, shorter than expected for a malaria infection and would be addressed in future studies.
From our results on the interaction of CQ with \( p,p' \)-DDT and \( p,p' \)-DDE, it was important to assess the residual DDT and DDE variable and how much of residual \( p,p' \)-DDT and/or \( p,p' \)-DDE would enter into or remain in the different compartments (the RPMI media, erythrocytes and infected erythrocytes) over time. In combination with liquid-liquid extraction, we developed a sensitive GC-MS analyses method and a novel HPLC-UV analysis method for measuring DDT and DDE levels in malaria culturing blood and media. Whilst the HPLC-UV method was relatively cheaper, faster, and effective in determining high DDT and DDE concentrations, the optimised GC-MS method proved to be effective in detecting levels as low as 78 pg/mL (ppt) DDE and 7.8 ng/mL (ppb) DDT in biological media. Using both the HPLC and GC-MS methods we observed that malaria parasites influence distribution of the compounds between the erythrocytic and media fractions. *P. falciparum* D10 infection at ±10% parasitemia lead to much faster equilibration (less than 8 hours) between compartments. Equimolar distribution of \( p,p' \)-DDE was observed, but the parasites lead to trapping of the largest fraction of \( p,p' \)-DDT in the erythrocyte compartment. These results indicate that a substantial amount would reach the intra-erythrocytic parasite and could influence the parasite directly, possibly leading to either synergistic or antagonistic drug interactions.

This study is the first to illustrate the “good and bad” of the insecticide DDT in terms of CQ resistance and sensitivity toward the human malaria parasite *P. falciparum*. These results will hopefully have an important influence on how future policies on malaria control and treatment particularly in endemic areas will be addressed and could also have an impact on the anti-malarial drug discovery approach.
Opsomming

Dichlorodifenieltrichloroetaan (DDT) is op groot skaal in landbouplaagbeheer gebruik en word nog steeds gebruik vir binnenshuisse oppervlakbespuiting om malaria te beheer. Die lipofilisiteit van DDT en sy afbraakprodukt dichlorodifenieldichloroetileen (DDE) dikteer dat hulle met membrane, lipiede en hidrofobiese proteïene in die biologiese omgewing associeer. Stadige afbraak veroorsaak dat DDT en DDE vir dekades in die omgewing agterbly, asook in individue wat in kontak is, of was met die insekdoder. In baie lande het gesinkroniseerde weerstand van die muskietvektor teenoor insekdoders en die malariaparasiet teenoor antimalarialmiddels geleidelik tot 'n drastiese styging in malariagevalle en tot malaria-epidemies. In hierdie studie word die invloed van lae vlak blootstelling van DDT en DDE op chlorokien (CQ) weerstand van die mens malariaparasiet, *Plasmodium falciparum*, geëvalueer.

Die *in vitro* aktiwiteit van *p,p'*-DDT en *p,p'*-DDE teenoor die bloedstadia van chlorokiensensitiewe (CQS) *P. falciparum* D10 en chlorokien-weerstandbiedende (CQW) *P. falciparum* Dd2 is bepaal deur gebruik te maak van twee komplementêre *in vitro* toetses (Malstat en SYBR Groen toetses). Die 50% inhibisie konsentrasies (IC₅₀s) van *p,p'*-DDT en *p,p'*-DDE is bepaal as ±14 to 38 µM (5-12 µg/mL) en was hoogs vergelykbaar tussen CQS en CQW *P. falciparum* stamme. Hierdie resultaat het aangedui dat die proteïene betrokke by CQ weerstand geen effek op die aktiwiteit van die insekdoder DDT en die afbraakprodukt DDE het nie.

Om die invloed van DDT en DDE op CQ aktiwiteit te evalueer, is die aktiwiteit van kombinasies van die verbindings in vaste verhoudings getoets, tesame met isobologram ontleiding. Ons het gevind dat CQ sinergisties saam met *p,p'*-DDT en *p,p'*-DDE teen CQS *P. falciparum* D10 werk. Daarteenoor het beide *p,p'*-DDT en *p,p'*-DDE antagonistiese werking getoon teenoor CQ aktiwiteit met CQW *P. falciparum* Dd2 as teiken. Dit het aangedui dat *p,p'*-DDT en *p,p'*-DDE wel 'n invloed op CQ weerstand het of 'n aktiwiteit van CQ, anders as hemozoin polimerisasie, beïnvloed. Die waarneming van resiproke sinergisme en antagonisme van *p,p'*-DDT en *p,p'*-DDE in kombinasie met CQ teenoor die CQS D10 en CQW DD2 stamme respektiewelik, is hoogs betekenisvol en dui op seleksie van CQ-weerstandige stamme in die teenwoordigheid van *p,p'*-DDT en *p,p'*-DDE. Mense wat lae vlakke van sirkulerende DDE/DDT het, het dus 'n hoër risiko om CQW malaria te kry.
Verder is gevind dat medium termyn (nege dae) DDE blootstelling van CQS *P. falciparum* D10 nie weerstand nie veroorsaak nie, want geen beduidende verandering in die aktiwiteit van CQ, *p,p*-DDT en *p,p*-DDE teenoor die bloed stadiums van die CQS stam is waargeneem nie. Hierdie blootstelling is egter korter as in 'n malaria-infeksie en sal verder bestudeer word in toekomstige studies.

Vanuit die interaksie resultate van CQ met *p, p*-DDT en *p, p*-DDE was dit belangrik om die residuele DDT en DDE veranderlike te evalueer, asook die distribusie van *p,p*-DDT en *p,p*-DDE tussen die verskillende kompartmente (die kultuurmedium, eritrosiete en geïnfecteerde rooibloedselle) oor verloop van tyd. In kombinasie met vloeistof-vloeistof ekstraksie, het ons 'n sensitiewe GC-MS en nuwe HPLC-UV analysemethode ontwikkel vir die meet van DDT en DDE-vlakke in bloed (normale en geïnfecteerde eritrosiete) en die kultuurmedium. Terwyl die HPLC-UV metode relatief goedkoper, vinniger en effektief in die bepaling van hoë DDT en DDE-konsentrasies is, was die geoptimaliseerde GC-MS metode doeltreffend in die opsporing van vlakke so laag as 78 pg/mL (dpt) DDE en 7.8 ng/mL (dpb) DDT in biologiese media. Met behulp van beide die HPLC-UV en GC-MS metodes is waargeneem dat die malariaparasiet die ekwilibrasie van die verbinding tussen die eritrosiet- en media kompartmente beïnvloed. *P. falciparum* D10 infeksie met ± 10% parasitemia lei tot vinniger ekwilibrasie (minder as 8 uur) tussen die kompartmente. Ekwimolêre verspreiding van *p,p*-DDE is waargeneem, maar die parasiete het die grooste fraksie van *p,p*-DDT in die eritrosiet kompartment vasgevang. Hierdie resultate wys dat 'n aansienlike fraksie die intra-eritrositiese parasiet kan bereik en sodoende die parasiet direk kan beïnvloed en moontlik kan lei tot sinergistiese of antagonistiese middel interaksies.

Hierdie studie is die eerste om die "goed en sleg" van die insekdoder DDT in terme van CQ weerstand en sensitiwiteit teenoor die menslike malariaparasiet *P. falciparum* te illustreer. Hierdie resultate sal hopelik 'n belangrike invloed hê op die toekomstige beleid oor die beheer van malaria en behandeling, veral in endemiese gebiede, en mag ook 'n impak hê op die antimalariamiddel navorsing.
Dedicated to my loving mom and brothers for their love, patience and support
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<tr>
<td>ABC</td>
<td>ATP binding cassette</td>
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<td>ACN</td>
<td>acetonitrile</td>
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<tr>
<td>DDD</td>
<td>dichloro-diphenyl-dichloroethane</td>
</tr>
<tr>
<td>DDE</td>
<td>dichloro-diphenyl-dichloroethylene</td>
</tr>
<tr>
<td>DDT</td>
<td>dichloro–diphenyl-trichloroethane</td>
</tr>
<tr>
<td>DELI</td>
<td>double-site enzyme-linked LDH immunodetection</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECD</td>
<td>electron capture detector</td>
</tr>
<tr>
<td>EI</td>
<td>electronic impact ionisation</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>FAS II</td>
<td>fatty acid biosynthesis II</td>
</tr>
<tr>
<td>FCM</td>
<td>flow cytometry</td>
</tr>
<tr>
<td>FIC</td>
<td>fractional inhibition concentration</td>
</tr>
<tr>
<td>FIC&lt;sub&gt;index&lt;/sub&gt;</td>
<td>fractional inhibition concentration index</td>
</tr>
<tr>
<td>GC</td>
<td>gas chromatography</td>
</tr>
<tr>
<td>GC-ECD</td>
<td>gas chromatography- electron capture detector</td>
</tr>
<tr>
<td>GMEP</td>
<td>Global Malaria Eradication Program</td>
</tr>
<tr>
<td>GMM</td>
<td>genetically modified mosquitoes</td>
</tr>
<tr>
<td>GS</td>
<td>gramicidin S</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>HPLC–MS</td>
<td>high performance liquid chromatography linked mass spectrometry</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>HPLC-UV</td>
<td>high performance liquid chromatography linked ultraviolet spectrophotometry</td>
</tr>
<tr>
<td>HS</td>
<td>headspace</td>
</tr>
<tr>
<td>HS-SPME</td>
<td>headspace-solid phase microextraction</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>50% growth inhibition concentration</td>
</tr>
<tr>
<td>IC&lt;sub&gt;max&lt;/sub&gt;</td>
<td>maximum inhibitory concentration</td>
</tr>
<tr>
<td>IDL</td>
<td>instrument detection limit</td>
</tr>
<tr>
<td>IRS</td>
<td>indoor residual spraying</td>
</tr>
<tr>
<td>ITNs</td>
<td>insecticide treated bed nets</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>kdr</td>
<td>knock down resistance</td>
</tr>
<tr>
<td>LLE</td>
<td>Liquid–liquid extraction</td>
</tr>
<tr>
<td>LLITN</td>
<td>long lasting insecticide treated bed nets</td>
</tr>
<tr>
<td>LOD</td>
<td>limits of detection</td>
</tr>
<tr>
<td>LOQ</td>
<td>limits of quantification</td>
</tr>
<tr>
<td>LPME</td>
<td>liquid phase microextraction</td>
</tr>
<tr>
<td>m/z</td>
<td>mass charge ratio</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>MS/MS</td>
<td>tandem mass spectrometry</td>
</tr>
<tr>
<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NBT</td>
<td>nitro blue tetrazolium</td>
</tr>
<tr>
<td>NCI</td>
<td>negative chemical ionization</td>
</tr>
<tr>
<td>OCPs</td>
<td>organochlorinated pesticides</td>
</tr>
<tr>
<td>P</td>
<td>significance probability</td>
</tr>
<tr>
<td>P. berghei</td>
<td>Plasmodium berghei</td>
</tr>
<tr>
<td>P. falciparum</td>
<td>Plasmodium falciparum</td>
</tr>
<tr>
<td>P. knowlesi</td>
<td>Plasmodium knowlesi</td>
</tr>
<tr>
<td>P. malariae</td>
<td>Plasmodium malariae</td>
</tr>
<tr>
<td>P. ovale</td>
<td>Plasmodium ovale</td>
</tr>
<tr>
<td>P. vivax</td>
<td>Plasmodium vivax</td>
</tr>
<tr>
<td>PCBs</td>
<td>polychlorinated biphenyls</td>
</tr>
<tr>
<td>PES</td>
<td>phenazine ethosulfate</td>
</tr>
<tr>
<td>PfATP-6</td>
<td><em>P. falciparum</em> adenosine triphosphatase 6</td>
</tr>
<tr>
<td>PfCRT</td>
<td><em>P. falciparum</em> chloroquine resistance transporter</td>
</tr>
<tr>
<td>PfDHFR</td>
<td><em>P. falciparum</em> dihydrofolate reductase</td>
</tr>
<tr>
<td>PfMDR-1</td>
<td><em>P. falciparum</em> multidrug resistance protein-1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>pLDH</td>
<td>parasite lactate dehydrogenase</td>
</tr>
<tr>
<td>ppb</td>
<td>parts per billion</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>ppt</td>
<td>parts per trillion</td>
</tr>
<tr>
<td>QC</td>
<td>quality control</td>
</tr>
<tr>
<td>R²</td>
<td>coefficient of determination</td>
</tr>
<tr>
<td>RBC</td>
<td>red blood cell</td>
</tr>
<tr>
<td>RPMI media</td>
<td>Roswell Park Memorial Institute media</td>
</tr>
<tr>
<td>SAGM</td>
<td>saline-adenine-glucose-mannitol</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SIM</td>
<td>selected ion monitoring</td>
</tr>
<tr>
<td>SPE</td>
<td>solid-phase extraction</td>
</tr>
<tr>
<td>SPME</td>
<td>solid phase microextraction</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>tR</td>
<td>retention time</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>USAID</td>
<td>United States of America Agency for International Development</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>v/v</td>
<td>volume to volume ratio</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
<tr>
<td>WHOPES</td>
<td>WHO Pesticides Evaluation Scheme</td>
</tr>
</tbody>
</table>
Preface

The coincidental resistance of malaria parasites to antimalarial drugs and insecticides to the mosquito vector has led to a drastic increase in the number of cases and deaths by malaria in several countries. The resistance towards these small organic molecules in the malaria parasite has been linked to membrane transporter proteins that confer resistance by expelling the drug from the target environment. In *Plasmodium falciparum*, CQ resistance has been mapped onto mutations in the genes coding for the *P. falciparum* CQ resistance transporter protein (*PfCRT*) and multidrug resistant protein (*PfMDR-1*), as has been the case with other antimalarial drugs. The resultant multiple resistance profile of some of the transporters indicated that they can accommodate a wide spectrum of organic molecules, many of which contain a phenyl-chloride moiety. The insecticide dichloro–diphenyl-trichloroethane (DDT) and its major break down product dichloro-diphenyl-dichloroethylene (DDE) have two such moieties. A question of whether organochlorines such as DDT and DDE can place selective pressure on possibly the more resistant malaria strains that can cope with DDT and/or DDE was raised. Could the selection for malaria strains that can pump the slightly more polar DDE out of the cytoplasm into the food vacuole or even out of the parasite into the erythrocytic cytoplasm lead to associated broad spectrum resistance?

We hypothesize that lipophilic DDT and DDE may associate primarily with membranes; however degradation of DDT may cause the parasite to respond in a way that may lead to resistance towards non-related drugs. Similar scenarios are also possible for other small organic aromatic or heterocyclic compounds such as other pesticides, food dyes, detergents and preservatives that may be abundant in an infected individual’s blood.

In this pilot study we report the investigation of the influence of DDT and DDE on CQ resistance in *P. falciparum*. The overall goal of this MSc project was to assess if low levels of the insecticide DDT and its metabolite DDE have an influence in the selection of *Plasmodium falciparum* strains resistant to conventional antimalarial prophylactics. The following objectives were set to achieve this goal:

- Development of an HPLC-UV method and sensitive GC-MS method to determine the levels of DDT and/or DDE in blood and malaria culture medium, as well as in *P. falciparum* infected culture samples (*Chapter 2*);
• Determining the *in vitro* influence of DDT and DDE on the viability of *P. falciparum* (CQR and CQS strains) using standard complementary dose-response assays (Malstat and SYBR Green 1 assays) (*Chapter 3*);

• Assessing the influence of DDT and DDE exposure on CQ resistance (*Chapter 4*):
  
  − by determining the *in vitro* drug interactions of DDT and DDE in combination with CQ on viability of *P. falciparum* CQR and CQS strains,
  
  − growing the *P. falciparum* CQR and CQS strains at inhibitory levels of DDE and determining CQ sensitivity
  
  − determining the DDE and DDT distribution in malaria cultures (RPMI medium vs infected erythrocyte and erythrocytes) grown in the presence of DDE using appropriate methods developed in *Chapter 2*.

In *Chapter 1*, an overview of the impact of antimalarial drugs and insecticides in malaria control is given. The next three chapters address each of the experimental objectives as described above, with the results obtained in each case fully discussed. These chapters were written in the form of articles for ease in future publication. Repetition was inevitable; however, it was kept to a minimum. The final chapter, *Chapter 5*, summarises the most important results and conclusions in this study.
CHAPTER 1

Literature Review: Impact of antimalarial drugs and insecticides on malaria control

1.1 Malaria background

Malaria is one of the leading infectious diseases in sub-Saharan Africa which mostly affects children and the vulnerable in a society (WHO 2010a, WHO 2010b). The humid climate in the region promotes breeding and development of the mosquito vector and its parasite respectively (Odebayo and Krettli 2011). The parasitic infection in humans is caused by transmission of the Plasmodium species by an infected Anopheline mosquito through a mosquito bite to an uninfected individual (Bray and Garnham, 1982; Hay et al., 2004).

There are five major Plasmodium species that affect humans which are P. falciparum, P. vivax, P. malariae P. ovale and P. knowlesi (Greenwood et al., 2008). P. knowlesi was most recently found to also affect humans and reported to be potentially life threatening (Cox-Singh et al., 2008). All five species together led to the occurrence of roughly 225 million cases annually worldwide in 2009, resulting in 781 000 recorded deaths (WHO 2010a). P. falciparum and P. vivax infections cause the majority of malaria cases (Guerin et al., 2002). Whilst malaria caused by P. falciparum is most prevalent globally and particularly in Africa, P. vivax malaria is mostly found in Asia and South America (Bray and Garnham 1982; Price et al., 2007; Guerin et al., 2002).

Although malaria was successfully eradicated in most parts of the world, particularly subtropical regions, in the 1950s, the disease is re-emerging. Failure to control the disease through effective vector control and treatment regimes has therefore resulted in an increase in cases and deaths from malaria infection (Guerin et al., 2002; WHO 2010a). The international community is therefore urged to ensure sufficient and predictable global funding to meet malaria control targets set as part of the drive to reach the health-related Millennium Development Goals by 2015 (WHO 2010a). The World Health Assembly and Roll Back Malaria Partnership aim to reduce the numbers of cases and deaths recorded in 2000 by 75% or more by 2015 (WHO 2010a). Policies and strategies for malaria control set for 2015 are therefore aimed at reaching all
persons at risk for malaria with insecticide-treated mosquito bed nets, indoor residual spraying, providing laboratory-based diagnosis for suspected cases of malaria, and effective treatment of all confirmed cases (WHO 2010a).

This chapter will focus on the currently employed and future malaria vector control and treatment strategies.

1.2 Malaria life cycle

As summarized in Figure 1.1, the human malaria parasite has two hosts, the mosquito and the human body. The malaria life cycle begins when a *Plasmodium* infected female *Anopheles* mosquito takes a blood meal from an uninfected human through a bite, and inoculates sporozoites into the host during the process (Amina et al., 2010). These sporozoites infect the human liver cells where they then mature into schizonts before being ruptured and released as merozoites (Ejigiri and Sinni 2009). The parasites multiply asexually, and the merozoites invade the red blood cells otherwise known as erythrocytes (Prudencio et al., 2006). Ring stage parasites mature into trophozoites, then schizonts which mature to form separate merozoites. The rupture of the erythrocyte releases merozoites for the cycle to repeat itself again in the erythrocyte (Bannister et al., 2000). It takes 48 hours to 72 hours for reinvasion of another erythrocyte in *P. falciparum* and *P. malariae* respectively (Amina et al., 2010). Some of the parasites develop into gametocytes (Amina et al., 2010).

Male and female gametocytes are ingested by the mosquito during a blood meal. (Baton and Ranford-Cartwright 2005; Mueller et al., 2010). The parasites multiply in the mosquito in a cycle known as the sporogonic cycle. The male gametocytes penetrate the female gametocytes in the mosquito’s stomach, forming zygotes. These zygotes become motile and elongated (ookinetes), and invade the mid-gut of the mosquito where they develop into oocysts. The oocysts grow, rupture and release sporozoites which go to the mosquito’s salivary glands before being inoculated into a new human host and the cycle starting all over again (Baton and Ranford-Cartwright 2005, Mueller et al., 2010).
1.3 Malaria control and treatment

It has been reported that few African countries have been able to rapidly scale up malaria diagnostic testing at national level, allowing for the implementation of timely and accurate surveillance of the disease (WHO 2010a).

Over the years, different strategies have been implemented in a bid to fight against malaria. Amongst these strategies are control measures, aimed at the exclusion of new illnesses and deaths caused by malaria without necessarily blocking spread of the disease. Another strategy is aimed at eliminating malaria and stopping the spread of the disease in a smaller area such as in a single country. Eradication is the last strategy which involves total elimination of the disease on a global level (Greenwood 2008).
Malaria was successfully controlled and eliminated in other parts of the world such as Western Europe and the United States of America (USA) in the first half of the 20th century (Amina et al., 2010). The World Health Organisation (WHO) then launched a Global Malaria Eradication Program (GMEP) for the first time in 1955 as a result of the previously recorded success in Western Europe and the USA (Spielman et al., 1993). The agricultural insecticide, dichlorodiphenyl-trichloroethane (DDT) was successfully used for agricultural pest control worldwide (Hay et al., 2004) and it had already been used successfully in other parts of the world to eradicate the malaria. DDT was then employed to prevent transmission of the disease via its mosquito vector in areas still affected by this disease. To complement the DDT in malaria eradication, chloroquine (CQ) was used as the major drug in the malaria treatment program (Hay et al., 2004). This strategy was initially successful; however, drug and insecticide resistance then emerged resulting in a spike in malaria cases and deaths (Najera et al., 2011; Trape 2001; and Clyde and Shute 1957). Unfortunately, sub-Saharan Africa was not included in this initial eradication program, therefore this contributed to the spread and major prevalence of the disease in this part of the world (WHO 2010a).

As a background to our study on DDT and CQ in the malaria parasite sensitivity and resistance, malaria control using antimalarial drug treatment, mosquito vector control using insecticides, other methods used to control malaria, as well as drug and insecticide resistance, are discussed further in following sections of this chapter.

1.3.1 Malaria control

1.3.1.1 Vaccine development

Genetically modified vaccines to prevent transmission of malaria have been and are still under investigation to minimise infection and death of children by the disease (Greenwood et al., 2008). Vaccines currently under development are placed in three categories based on the stage of infection. Pre-erythrocytic stage vaccines, most of which were already abandoned in clinical trials because of their short lasting immunity, function by preventing infection to the human hosts and thus later progression to the disease (Stoute et al., 1997; Bojang et al., 2001). Asexual blood stage vaccines act by preventing the disease but not the infection, for example VAR2CSA which has the unfortunate side effect of binding placental chondroitinsulphate in pregnant
women (Gill et al., 2009). Transmission blocking vaccines, for which studies are still underway, help to reduce transmission of the parasite in the community without necessarily directly protecting the vaccinated individual (Greenwood et al., 2008).

1.3.1.2 Transgenic mosquitoes
As a result of previous failures of existing methods in malaria vector and parasite control due to continued emergence of drug and insecticide resistance respectively, and renewed efforts to find an effective vaccine, genetically modified mosquitoes (GMM) were then investigated and developed in another effort to fight against malaria. These transgenic mosquitoes would either reduce vector population sizes or replace existing vectors with those unable to transmit the disease (Marshal and Tailor 2009). Mosquitoes that confer resistance to rodent malaria have already been successfully engineered although more research still needs to be done since transgenic mosquitoes may become the future in malaria control and eradication (Ito et al., 2002). However, the global and even local release of transgenic mosquitoes is hampered because of the controversial nature of GM strategies.

1.3.2 Current status of antimalarial drug treatment
1.3.2.1 Antimalarial drug treatment
There are currently seven classes of antimalarial drugs, based on their chemical structures, being used as prophylactics and to treat malaria (Amina et al., 2010). One of the most widely used classes is the 4-aminoquinones which have been shown to inhibit hemozoin polymerization in the parasite food vacuole by forming complexes with ferrirprotoporphirine IX. Chloroquine (CQ) and amidaquine fall within this class of drugs, and are the most frequently used (O’Neill et al., 2006).

The second class, the arylaminoalcohols, also inhibit hemozoin formation outside the food vacuole by preventing entry of hemoglobin into the digestive vacuole. Quinine and mefloquine are the most commonly used drugs in this class which is characteristic of its chirality (Amina et al., 2010). These drugs are generally administered in racemate form (Amina et al., 2010). Combinations of quinine with tetracycline, doxycycline or clyindamycin, and mefloquine with artesunate are usually recommended for a more pronounced effect on the parasite (Brocks and Mehvar 2003).
Antifolates fall in a third class of drugs that inhibits biosynthesis of tetrahydrofolate (Yuthavong et al., 2006). This class includes the sulphonamides, sulphadoxine and sulphonedapsone, that inhibit dihydropteroate synthase whilst others such as pyrimethamine, cycloguanil and chlorecycloguanil inhibit dihydrofolate reductase. Combinations within this class led to sulphadoxine-pyrimethramine treatments that were used to replace CQ treatment after CQ resistance emerged in the 1960’s.

Primaquine is the only drug in the 8-aminoquinolines class studied thus far that inhibits the pentose phosphate cycle in liver (sexual and pre-erythrocytic) stage parasites (Amina et al., 2010). It is used to treat both P. falciparum and P. vivax malaria (Vale et al., 2009), and is currently one of the few drugs used to treat liver stage malaria.

Proguanil and atovaquone lie within the class of “inhibitors of the respiratory chain”. These drugs act by inhibiting the mitochondrial electron transport chain in treating uncomplicated P. falciparum malaria (Sabchareon et al., 1998). Antibiotics such as clindamycin and doxycycline inhibit prokaryote-like protein biosynthesis resulting in weakened parasite maturation (Stanway et al., 2009).

One of the most recent drug classes that entered the global arena of malaria drugs is the artemisinins. Although the mechanism of action of artemisinins is not yet well understood, they are known to inhibit the endoplasmic reticulum of the P. falciparum adenosine triphosphatase calcium pump (PfATP-6) and also target food vacuole proteins (Amina et al., 2010). Most countries in endemic areas now have adopted artemisinin combination treatments (ACTs) as a strategy to limit resistance (Whitty et al., 2008). Artemether and artesunate are amongst the most common derivatives in clinical use in this class of drugs. They are both modified from the hemiacetale form of artemisinin, dihydroartemisinin (Mercer 2009).

As reviewed by Schlitzer (2008), several new antimalarial drugs and drug combinations are under investigation (Schlitzer 2008). Piperaquine-dihydroartemisinin and pyronaridine-arthesunate combinations and tafenoquine (a primaquine derivative) are in the advanced stages of clinical trial studies. Clinical data is already available for azithromycin, pafuramidine, fosmidomycin and dapsone-chlorproguanil-arthesunate new trial drugs (Amina et al., 2010). T3,
AQ-13, ferroquine and tert-butyl-isoquine are amongst the new drugs beginning clinical evaluation. Artemisone, OZ-277 and GW844520 on the other hand have been dropped out due to bioavailability in malaria patients being only a third of the values obtained with healthy volunteers for OZ-277, and unexpected cardiotoxicity being observed in dogs with GW844520 use (Schlitzer 2008; Amina et al., 2010). Although antimalarial drug research is very active, there is an urgent need for new drugs as resistance to all the current drugs in clinical use have already been observed as discussed below.

1.3.2.2 Antimalarial drug resistance

Amongst the factors resulting in antimalarial drug resistance are, the parasite mutation rate, the overall parasite load, the strength of the drug chosen for treatment, compliance to the treatment, and the fitness costs associated with resistance mutations (Petersen et al., 2011). In vitro studies on Southeast Asia isolates showed an accelerated resistance to multiple drugs (ARMD) phenotype in which a very high mutation rate facilitates adaptation of the parasite to a changed environment as a result of changing drug selection pressures (Sniegowski et al., 2000; Rathod et al., 1997). The observation of the emergence of resistance to new drugs in Southeast Asia is linked to possible association with the AMRD phenotype (Rathod et al., 1997).

Since the emergence of CQ resistance, the use of CQ against *P. falciparum* parasites has been greatly minimized (Hayton and Su 2008). CQ has remained effective against other *Plasmodium* strains. However, CQ resistance in *P. vivax* strains has recently been reported in South Eastern Asia (Baird et al., 2007). Amodiaquine has effectively been used as an alternative to low CQ resistant (CQR) parasites although amodiaquine resistance has also been noted in several parts of Asia (O’Neill et al., 2006; Sa et al., 2009). Resistance to CQ and other drugs within its class is conferred to by mutation of the *P. falciparum* chloroquine resistance transporter (*PfCRT*) gene which lies on the membrane of the parasite’s food vacuole. The “pump” acts by expelling the drug(s) out of the digestive vacuole, thus preventing the formation of CQ-heme complexes and eventual toxic build up of heme in the vacuole which is supposed to aid in the killing of the parasite (Amina et al., 2010). As a way of overcoming CQ resistance in *P. falciparum* strains sulphadoxine-pyrimethamine combination therapy was used to replace CQ (Yuthavong et al., 2006).
Although little is known about the tertiary structure of PfCRT, bioinformatics analysis has predicted it to be in the drug/metabolite transporter family with 10 transmembrane domains, with both the C- and N- termini facing the cytosolic side of the organelle (Martin and Kirk 2004). Amino acids, weak bases and positively charged organic ions are amongst some of the substrates of the drug/metabolite transporter family (Martin and Kirk 2004). The K76T (Lys76 to Thr76) mutation in PfCRT confers CQ resistance by removing a positively charged residue from the transmembrane domain 1 in a putative pore, and replacing it with an uncharged amino acid providing a route for the diprotomated CQ to escape the digestive vacuole (Martin and Kirk 2004).

The *P. falciparum* multidrug resistance protein-1 (PfMDR-1) membrane associated transport protein is responsible for drug resistance in the arylaminoalcohols class of antimalarial drugs (Amina *et al*., 2010). This “pump” allows entry of arylaminoalcohols into the digestive vacuole where they are ineffective. It is found in Asian countries where resistance to mefloquine and quinine is mostly experienced (Pickard *et al*., 2003). The PfMDR-1 has also been linked to CQ resistance in Africa, and a functional relationship between PfCRT and PfMDR-1 proteins has been suggested (Hastings 2006; Barnes *et al*., 1992; Osman *et al*., 2007). Cross resistance between CQ and amodiaquine has been reported and linked to both the PfCRT and PfMDR-1 proteins (Sa 2009; Petersen *et al*., 2011).

The PfMDR-1, like PfCRT also sits on the digestive vacuole membrane of the parasite (Cowman *et al*., 1991). It was originally identified using a candidate gene approach. A gene encoding an ATP binding cassette (ABC), with 12 helices across the membrane and C- and N- termini expected to extrude into the cytosol, was identified (Foote *et al*., 1989; Durasingh and Cowman 2005). PfMDR-1 pumps drugs into the digestive vacuole, diverting them from their target sites in the cytoplasm (Durasingh and Cowman 2005).

A third transporter protein associated with drug resistance is the multidrug resistance associated protein (PfMRP) which belongs to the ABC transporter family (Klokouzas *et al*., 2004). It is, however, not a major determinant in resistance, but modifies drug responses (Raj *et al*., 2009). It was hypothesized that it works in collaboration with other transporters to expel drugs and other metabolites from the parasite (Raj *et al*., 2009).
Multiple mutations in the *P. falciparum* dihydrofolate reductase (*Pf*DHFR) gene cause resistance in the antifolate class (Amina *et al*., 2010). The triple mutant form is inactive for pyrimethamine though it is sensitive for chlorcycloquanil. Dihydrofolate inhibitors have on the other hand been shown to be ineffective against the quadruple mutant form of antifolates (Hankins *et al*., 2001).

Artemisinin resistance is linked to mutation in the *P. falciparum* ATP-6 (*Pf*ATP-6) gene (Jambou *et al*., 2005). Higher doses of primaquine have been reported to overcome artemisinin resistance which was reported to occur under very rare circumstances (Bunnag *et al*., 1994). Most antimalarial drugs are now being administered in combination with each other, all having different target mechanisms within the parasite, to decrease the emergence of drug resistance (Petersen *et al*., 2011). Atovaquone-proguanil (AP) combinations have been used with success to reduce emerging resistance to inhibitors of the respiratory chain (Sabchareon *et al*., 1998) and no AP resistant strains have been identified as yet (Amina *et al*., 2010).

Ultimately, only artemisinin based combinations with drugs from other classes have proven to be most reliable and recommended for treatments (Amina *et al*., 2010). Currently, ACTs being used are artemether with lumefantrine (Co-artem), dihydroartemisinin with piperaquine and, artesunate in combination with mefloquine, amodiaquine, pyronaridine, and sulphadoxine-pyrimethamine, respectively (WHO 2010a).

**1.3.3 Current status of malaria vector control**

### 1.3.3.1 Malaria vector control

Based on the WHO Pesticides Evaluation Scheme (WHOPES), there are currently four classes of insecticides available for indoor residual spraying (IRS) programmes in malaria control (Walker *et al*., 2003). These are carbamates, organochlorines, organophosphates and pyrethroids (Walker *et al*., 2003). Most new malaria control interventions are however focusing on pyrethroids since they are the only class of insecticides approved by the WHOPES for long lasting insecticide treated bednets (LLITN) (Zaim *et al*., 2000). Because of the major risk of pyrethroid resistance by the mosquito, this may have detrimental effects on malaria control activities particularly in West and Southern Africa (WHO 2005; Pinto *et al*., 2007; Hargreaves *et al*., 2000; Casimiro *et al*., 2006).
Insecticide treated bednets (ITNs) and indoor residual spraying (IRS) programs have been, and are currently being used as strategic means to control the Anopheline mosquito vector (Guyatt et al., 2002). According to the World Malaria Report 2010, ITNs that were set to be distributed in sub-Saharan Africa between 2008 and 2010 were meant to protect 578 million people. However, only 10% of the 765 million African population at risk of malaria infection was protected from malaria infection through IRS in 2009 alone (WHO 2010a).

Pyrethroids which are also used internationally as agricultural pesticides replaced dichloro–diphenyl-trichloroethane (DDT) in the 1990’s in some countries (Ranson et al., 2011). Previously DDT was used successfully in malaria control programs for IRS and continues to be used in other parts of the world where it is approved by the WHO (Spielman et al., 1993, Walker et al 2003). Pyrethroids are also being increasingly deployed for IRS programmes in Africa (Zaim et al., 2000). Both DDT and pyrethroids have the same mechanism of action against the mosquito (Ranson et al., 2011). These insecticides both act by targeting a sodium gated channel involved in neuronal signal transmission in the mosquito (Ranson et al., 2011). Closing of the sodium gated channel is delayed due to insecticide binding, leading to prolonged action potential, thereby resulting in repetitive neuron firing, paralysis and eventual death of the mosquito (Ranson et al., 2011). The risk of resistance to both types of insecticides is therefore increased by virtue of them having the same mechanism of action (Davies et al., 2008).

New strategies are therefore being advanced to overcome the insecticide resistance (Amina et al., 2010). The mosquito’s molecular biology and the biochemistry of the blood it sucks from the human host are being studied to facilitate in the identification of specific attractant and repellent molecules (Amina et al., 2010). Broad based analysis of the mosquito and parasite genome are also being studied to gain a better insight into the mechanisms of parasite development in the mosquito (Greenwood et al., 2008).

1.3.3.2 Insecticide resistance

There are essentially five types of insecticide resistance mechanisms as described by Ranson et al. (2011). Target site resistance which is as a result of changes in the insecticide target site by “non-silent point mutations” is described below for DDT and pyrethroids. Metabolic resistance is caused by over-expression of enzymes resulting in an alteration of the enzyme’s affinity for the
insecticide (Ranson et al., 2011). Cytochrome P450 enzymes are primarily responsible for pyrethroid metabolism in insects, 111 such enzymes of which are found in *Anopheles gambiae* (*An. gambiae*) (Feyereisen 2005; Ranson et al., 2002). Three possible P450 genes were found to be repeatedly expressed in pyrethroid resistant *An. gambiae* mosquito populations (Djouaka 2008; Muller et al., 2007; Muller et al., 2008). Modifications in the mosquitoes’ cuticles resulting in reduced penetration of the insecticide causes cuticular resistance (Ranson et al., 2011). Behavioural resistance is as a result of changes in the mosquito’s behaviour which causes the mosquito to minimise or avoid toxic effects because of contact with the insecticides (Ranson et al., 2011). Cross resistance, which may also occur between insecticides from different classes, occurs when two different insecticides have their resistance conferred to through the same resistance mechanism (Ranson et al., 2011). Target site and metabolic resistance mechanisms have, however, been the most attributed to insecticide resistance in malaria control (Ranson et al., 2011).

Resistance to DDT and pyrethroids is conferred in the mosquito, to genetic mutations on the knock down resistance (*kdr*) gene (Matinez-Torrez et al., 1998). This has also been described as a result of cross resistance caused by the similar mechanisms of action and resistance of both types of insecticides (Williamson et al., 1996). Resistance to DDT and pyrethroids emerged as a result of extensive use of the compounds in agriculture (Davari et al., 2007). Pyrethroid resistant mosquitoes, *Anopheles funestus* (*An. funestus*) and *An. gambiae*, have been reported in some regions of Africa (Hargreaves et al., 2000; Girod et al., 2006).

Mozambique and South Africa are good examples of countries that showed direct failure of pyrethroid use to control malaria as a result of resistance to the insecticide (Ranson et al., 2011). Within four years of replacing DDT with the pyrethroid, deltamethrin, for IRS in the Kwa-Zulu Natal Province of South Africa, the number of cases and deaths by malaria increased four times between 1996 and the year 2000 (Brooke et al., 2001). Previously eradicated *An. funestus* re-emerged in South Africa and was found to survive in pyrethroid sprayed houses. This coincided with pyrethroid resistance being discovered in 1999. Through bioassays, the *An. funestus* species was found to be resistant to pyrethroids, but remained susceptible to DDT (Hargreaves et al., 2000). DDT was then reintroduced in South Africa in the year 2000 and this was followed by an
immediate and pronounced decline of 91% in the number of cases and deaths by malaria (Maharaj et al., 2005). Co-artem drugs and artemisinin combination therapy (ACT) were also introduced in the Kwa-Zulu Natal and Mpumalanga provinces of South Africa, respectively, helping to decrease the cases and deaths by malaria (O’Meara et al., 2010). Figure 1.2 from the South African Department of Health, shows the antimalarial drug-insecticide relationship used in relation to the number of cases and deaths by malaria in the country from 1971 to 2007.

![Figure 1.2](http://www.doh.gov.za/docs/reports/2007/malaria/part1.pdf)

**Figure 1.2** A descriptive relationship between antimalarial drugs and use of insecticides in controlling the number of cases and deaths by malaria in South Africa. This graph was adapted from the Department of Health, South Africa (http://www.doh.gov.za/docs/reports/2007/malaria/part1.pdf)

### 1.3.4 Antimalarial drugs and insecticides synergy in malaria control

The major aspects of the GMEP involved the use of chloroquine (CQ) as an antimalarial drug and dichloro-diphenyl-trichloroethane or DDT as an insecticide for vector control (Hay et al., 2004). This combination led to major successes in some parts of the world especially the developed countries that had sufficient resources to make the program a success (Petersen et al., 2011). In the 1960’s however, CQ resistance emerged as a result of mutations in the *P. falciparum* CQ resistance transporter (*PfCRT*) gene, spreading to different parts of the world.
DDT resistant mosquitoes also emerged leading to an increase in the number of cases and deaths by malaria (Najera et al., 2011; Trape 2001; Clyde and Shute 1957). As a result of the “failure” of the program to eradicate malaria from the world, the GMEP was then abandoned in 1969 (Amina et al., 2010).

Alternative antimalarial treatment in the form of sulphadoxine-pyrimethramine was then used to replace CQ, but unfortunately, mutated *P. falciparum* dihydrofolate reductase alleles conferring pyrimethamine resistance then emerged again (Hayton and Su 2008). Delivery of artemisinin derivative drug-based treatments and improvement of insecticide-based measures were then prompted as a way of finding new tools to control malaria (Amina et al., 2010).

### 1.4 Dichloro-diphenyl-trichloroethane

**1.4.1 The rise and fall of DDT**

From being the most utilised pesticide and insecticide all over the world during the World War II era, to being a disaster to wildlife and the environment, DDT fell from its miracle rankings and was banned because of widespread environmental contamination and links to death of wildlife, as well as possible detrimental human health effects.

The use of DDT was banned in the 1970’s in most parts of the world due to the detrimental effects caused by the organochlorine compound on the environment and possible negative effects on humans (Leber and Benya 1994; Dunlap 1981). The decision was informed after studies in the United States of America (USA) where extensive use of DDT was practiced through particularly agriculture and malaria vector control, showed that severe persistent environmental contamination occurred as a result of continued use of DDT (Walker et al., 2003). Publication of Rachel Carson’s Silent Spring in 1962 showed the negative effects of DDT on wildlife and prompted public concern on the safety of the compound in the environment (Walker 2003). Adverse human health effects associated with DDT have also been reported to date (Beard 2006). Amongst these are reproductive disorders and abnormalities, impaired bone mineral function, endocrine conditions, hormonally sensitive cancers, pancreatic, and other cancers, neurological impairments, and suppressed immune function, as discussed further by Beard (2006).
With compliance from 91 countries worldwide, the Stockholm Convention on Persistent Organic Pollutants in 2001 listed DDT as one of the 12 toxic chemicals to be eventually eliminated from the world (UNEP, 2002). However, as a result of DDT's successful use in eradicating the disease in other parts of the world during the GMEP era, the WHO listed it as one of the 12 insecticides for use only in vector control through IRS programs, particularly in malaria endemic and pandemic areas of the world such as sub-Saharan Africa (WHO 2007; Rogan and Chen 2005). DDT was then reintroduced in other countries such as South Africa and had a tremendous impact in malaria control as described earlier (Jaga and Dharmani 2003).

1.4.2 DDT and its metabolites

Regular DDT preparations consist mainly of \( p,p' \), \( o,p' \) and \( o,o' \) isomers which are metabolized by the liver into dichloro-diphenyl-dichloroethane (DDD), dichloro-diphenyl-acetate (DDA), and dichloro-diphenyl-dichloroethylene (DDE) (Figure 1.3). DDD, the \( o,p' \) derivative of DDT (De Francia et al., 2006), and DDE accumulate in fat tissue, whereas DDA is eliminated in urine and bile (Kitamura et al., 2002).

DDT and its major metabolite DDE, are however highly lipophilic in nature and persist for decades in all forms of life and the environment (Leber and Benya 1994; Spear 1999). DDE is reported to have a long half life of 7 to 11 years, but DDT and DDE concentrations in humans are believed to increase with age (Smith 2001; Wolff et al., 2000). In humans, DDT and DDE bioaccumulate mainly in the adipose tissue and to a lesser extent in breast milk and the bloodstream (Smith 1991; Ahlborg et al., 1995). DDT has been calculated to be eliminated from a human host after roughly 10 to 20 years, whilst DDE can stay in a human for as long as they live (Turusov et al., 2002).
1.4.3 DDT and DDE exposure in human tissue

High levels of human exposure to DDT and DDE through diet have been recorded (Jaga and Dharmani 2003). DDE, which is highly stable and non-biodegradable, is found in much higher concentrations in human tissue and tends to persist longer (Rogan and Chen 2005). Generally, the concentrations of DDT and its metabolites have been seen to decrease with decrease in DDT use and production (Rogan and Chen 2005). DDT concentrations in human tissues, however, remain high in areas where it has continued to be used such as some Asian countries, Africa, Mexico, Central and South America (Rogan and Chen 2005). Levels of DDT exposure and bioaccumulation in human tissue are discussed by Jaga and Dharmani 2003. It was challenging to make direct comparisons of DDT and DDE levels in human tissues between countries because of the different methods of detection and analysis of the compounds used, characteristics of exposure to DDT, studied groups and periods of study (Jaga and Dharmani 2003). Although blood serum concentrations are much lower than adipose tissue concentrations in humans, they give a reliable and good indication on the levels of exposure and/or contamination (Jaga and Dharmani 2003).

In South Africa for example, a median DDE concentration range in breast milk of between 5.2 and 7.7 µg/g was recorded in women from continuously sprayed areas compared to a median range of between 0.4 and 0.6 µg/g in women from areas of the same country where spraying with DDT was stopped (Bouwman et al., 1990). Most recently, DDT and DDE blood serum concentrations were determined in the rural Limpopo province of South Africa where people
were exposed to DDT through IRS. The sum of the mean of DDT was found to be 7.3 µg/g of lipid in serum (Van Dyk et al., 2010). Mean blood serum concentrations of DDE in the DDT sprayed areas in another South African study were 103 ± 85 µg/L (ppb) compared to unsprayed areas where the value was much lower, 6 ± 7 µg/L (Bouwman et al., 1991). Countries that have continued DDT use have recorded high DDT:DDE concentration ratios of close to 100% compared to 2-20% recorded in Europe and or the USA where use of DDT has been since stopped (Jaga and Dhamani 2003). Whilst high DDT:DDE ratios indicate chronic but ongoing exposure to DDT, a low ratio shows high environmental persistence and ongoing bioaccumulation (Attaran and Maharaj 2000).

A geometric total mean of 104.48 µg/g DDT was recorded for DDT sprayers in a Mexican study. These individuals were regularly exposed to DDT through IRS (Attaran and Maharaj 2000). In non-exposed individuals in the general populations in USA, Finland and Canada, the residual DDT was much lower (< 1 µg/g) in adipose tissue (Jaga and Dhamani 2003). The p,p’-DDE blood serum concentrations in DDT sprayers in another study was found to be 188 µg/L (118 ppb) (Yanez et al., 2002). Low level exposure of DDT and DDE in the developed world is as a result of the previously global use of DDT and its persistence in the environment (Jaga and Dharmani 2003).

1.4.4 Relationship between CQ and DDT

As is evidenced in several reports described earlier, CQ resistance only evolved after increased and probably uncontrolled exposure to the drug (Amina et al., 2010). The resultant multiple resistance profiles of some of the mutated transporters indicated that they can accommodate a broad spectrum of organic molecules (Amina et al., 2010). Many of the compounds accommodated contain a phenyl-chloride moiety. CQ, amodiaquine, lumefantrine, pyrimethamine, proguanil and cycloguanil are some of the antimalarial drugs from the different classes of drugs (Amina et al., 2010) that have this moiety. DDT and its major metabolite dichlorodiphenyldichloroethylene (DDE) contain two such moieties and can possibly be accommodated by the transporters (Figures 1.3 and 1.4). There has been no published study to our knowledge to determine the correlation between CQ and DDT/ DDE in terms of malaria.
parasite sensitivity and resistance or the direct effect(s) of DDT and DDE on erythrocytic stages of *P. falciparum* or any other malaria strains.

**Figure 1.4** Structural correlation between DDT and CQ

The overall goal of this project is to assess if exposure to DDT and DDE has an influence on the selection of *P. falciparum* strains resistant to chloroquine. Based on the background on levels of DDT and DDE exposure in the environment and in humans described above, it is therefore highly likely that there may be persistent low levels of the compounds in the malaria parasite as well that could aid in resistance selection.

### 1.5 References


http://www.dpd.cdc.gov/dpdx/HTML/Malaria.htm (Accessed 30 April 2011)


CHAPTER 2

*Development of analytical methods for the determination of p,p’-DDT and p,p’-DDE in biological media*

2.1 Introduction

Polychlorinated biphenyls (PCBs) and organochlorinated pesticides (OCPs) represent two of nine groups of organochlorinated compounds that were the subjects of the Stockholm Convention on Persistent Organic Pollutants (POPs) (UNEP, 2010). At the convention, a call was made for urgent global actions to reduce and eliminate releases of these compounds (UNEP, 2010).

Although dichloro-diphenyl-trichloroethane (DDT) was part of the list of POPs, it however continues to be used as the only organochlorine pesticide recommended by the World Health Organisation (WHO) for indoor residual spraying (IRS) to control malaria in several African and Asian countries where the disease is still endemic (UNEP, 2010; WHO, 2006). The Stockholm Convention allows the production and use of DDT for disease vector control only, provided that no safe, effective and affordable alternatives are locally available (UNEP, 2010). It was in 2006 that the World Health Organization (WHO) and the United States of America Agency for International Development (USAID) endorsed indoor DDT spraying to control malaria (WHO, 2006).

PCBs and OCPs enter the human body via the food chain or by respiration (Ballschmiter and Wittlinger, 1991; Ner’in et al., 1992). They have been detected in human tissues such as blood, (Burse et al., 1990; Rosell et al., 1993; Llu et al., 1994; Bucholski et al., 1996; Kanja et al., 1992), breast milk (Alawi et al., 1992; Bordet et al., 1993), fat (Bucholski et al., 1996; Kanja et al., 1992; Armishaw and Millar, 1993; Rivas et al., 1997; Unger et al., 1984) and urine (Martínez Vidal et al., 1998).

These compounds have also been detected in the environment, and trace amounts have been detected in our nutrition (Howe et al., 1990), thus posing a risk of causing adverse human health effects (López et al., 2007). Recent reports have raised the suspicion DDT and its metabolites
may be carcinogenic and mutagenic (Gonçalves and Alpendurada et al., 2002; Ye et al., 2006). These compounds have long half-lives as well as lipophilic properties which assist in their accumulation in adipose tissues (Garrido Frenich et al., 2000).

To estimate the potential health risk of these PCBs, OCPs and their metabolites, biological monitoring has been done in epidemiological studies, specifically in human blood (Cruz et al., 2003; Axmon et al., 2004; Bates et al., 2004; Apostoli et al., 2005). However, it has been argued that human blood serum does not provide as good an indicator as adipose tissue for monitoring organochlorine residues. Adipose tissue contains most of the lipophilic contaminants; it is therefore useful to also analyse recent or acute exposure in tissue biopsies and assessing the distribution of OCPs and PCBs (Moreno Frías et al., 2004).

Taking into consideration the clear difficulties in attaining human fat tissue samples to assess exposure to PCBs and other OCPs, blood is one of the most accessible media for ascertaining residual levels of OCPs. Blood is also the ideal medium for body burden estimation as close correlations between the concentrations of these compounds in blood and fat can be obtained (Garrido Frenich et al., 2000). Monitoring levels of DDT and its metabolites in blood and malaria culturing RPMI media in this study, however, was required to determine the contaminant variable in blood and malaria culture media.

In order to determine low concentration levels of these pesticides in biological media, efficient extraction and sample purification techniques, together with a final chromatographic determination are required (Martínez Vidal et al., 2000a). Liquid–liquid extraction (LLE) (Najam et al., 1999 and Rogers et al., 2004) or solid-phase extraction (SPE) by columns (Conka et al., 2005), C18 cartridges or disks (Pitarch et al., 2003; Covaci and Schepens, 2001) have been used to successfully extract PCBs and OCs from blood serum. Most of the reported procedures require subsequent clean up steps to eliminate interferences from the co-extracted bulk fatty matrix material. The laborious and time-consuming clean-up steps give cleaner extracts, but due to losses eventually lead to higher detection limits. Also, the risk of analytical error increases because of the incorporation of more steps in the sample preparation (López et al., 2007).
Solid phase microextraction (SPME) has most recently become a replacement for LLE and SPE. This method does not require solvents, but instead can be carried out directly from the liquid phase or from headspace (HS) over the liquid samples. It involves fewer steps and less sample handling (Pawliszyn, 1997). Only a few studies have been reported using SPME or HS-SPME for OCP determination in human serum (López et al., 2001; Beltran et al., 2001). Since it is difficult to directly determine the residual levels of pollutants in environmental samples with instrumental analysis due to their presence in minute levels, a sample pre-treatment procedure is necessary (Zhou Q et al., 2009). Thus far, sample pre-treatment methods for DDT and its main metabolites, such as solid phase extraction (Zhou Q et al., 2006; Zhou Q et al., 2007), solid phase microextraction (SPME) (Carvalho et al., 2008; Campillo et al., 2007), microwave-assisted extraction (Ji et al., 2007) and liquid phase microextraction (LPME) (Basheer et al., 2003) have been developed.

In studies conducted by Moreno Frías et al. (2004) the OCP and PCB containing sample extracts were cleaned by high performance liquid chromatography (HPLC), after liquid–liquid or solid–liquid extraction, and the first HPLC fraction was analysed by gas chromatography (GC) linked mass spectrometry (MS). Alternatively, after sample extraction and/or clean-up, the samples could be analyzed using capillary gas chromatography (GC) with an electron capture detector (ECD) (Najam et al., 1999; Rogers et al., 2004; Conka et al., 2005; Covaci and Schepens, 2001), mass spectrometry (MS) detection (Pitarch et al., 2003; Covaci and Schepens, 2001), high-resolution mass spectrometry with isotope-dilution quantification (IDHRMS) (Barr et al., 2003; Focant et al., 2004) or isotope dilution time-of-flight mass spectrometry (IDTOFMS) (Focant et al., 2004). GC-ECD has been the method of choice for analyses of PCBs and OCPs in human media (Luotamo et al., 1991; Sannino et al., 1996; Bennett et al., 1997; Rivas et al., 2001; Burse et al., 1990; Rosell et al., 1993; Bucholski et al., 1996; Rivas et al., 1997; Duarte-Davidson et al., 1991; Voogt et al., 1994). However, the high sensitivity of GC contrasts with the low specificity and lack of identification power of the ECD.

Retention time based identifications and determinations of pollutants, such as used with standalone HPLC and GC, are not regarded as being sufficient in current studies of such nature. Instead, tandem mass spectrometry (MS/MS) is used to confirm the presence of individual
contaminants (Garrido Frenich et al., 2000; Martínez Vidal et al., 2000b; Moreno Frias et al., 2001). GC linked mass spectrometry (GC-MS) with negative chemical ionization (NCI) (Rosen, 1987) is a selective approach particularly suitable for confirming the presence of organochlorinated compounds in environmental samples (Garrido Frenich et al., 2000). Electronic impact (EI) spectra usually contain sufficient structurally related fragment ions to allow absolute identification and, with the estimated uncertainty, ensure reliable results, to confirm the presence or absence of specific compounds in the analysis of biological samples (Martínez Vidal et al., 2002).

Several methods have of late been developed for the determination of DDT and its metabolites in environmental samples. Gas chromatography with electron capture detection (GC–ECD) (Zadora and Grochowalski, 2008; Hussen et al., 2007), HPLC with UV detection (Zhou Y et al., 2006; De Francia et al., 2006) and GC or HPLC–mass spectrometry (Valsamaki et al., 2006; Baugros et al., 2008) are amongst some of the mainly used analytical techniques.

GC–MS and HPLC–MS instrumentation are very expensive and the running costs are relatively high for general OCP and PCB analyses (Zhou Q et al., 2009). Although not the ultimate analytical technique, GC is generally used in OCP and PCB analyses. HPLC analysis is an alternative to GC analysis and an important tool for monitoring plasma levels of DDT and its metabolites (Zhou Q et al., 2009; Inouye et al., 1987; Benecke et al., 1987). Due to its stable sensitivity for DDT toxicokinetic studies, HPLC-UV has also been used for quantification of \( p,p' \)-DDT and \( p,p' \)-DDE in rat plasma, liver and brain (Tomiyama et al., 2000).

GC-MS has nowadays become the analytical tool for confirmatory analysis (Balinova, 1996). After separation the MS detection can confirm the correct mass and fragments that are generated in the MS or during MS/MS, provides information for structural clarification, which can be helpful in identifying unknown compounds (Garrido Frenich et al., 2000). GC-MS with an ion trap capability improves selectivity in the analysis of mixtures and therefore can lower detection limits by eliminating or minimizing chemical interferences (March and Tood, 1995, McLafferty, 1983; Busch et al., 1988; Lee et al., 1997; Pablos-Espada et al., 1999; Martínez Vidal et al., 1996).
This results in higher analytical sensitivity than single-stage MS analyses (Pablos-Espada et al., 1999; Martínez Vidal et al., 2000b; Johnson and Tost, 1985).

Chusaksri and colleagues (2006) proposed an MS fragmentation pattern for 2,4'- and 4,4'- DDT, DDD and DDE derivatives by using the positive ionisation mode for detection. After the capture of an electron, a stable carbocation is produced. As is depicted in Figure 2.1, it is proposed that the loss of the CHCl₂ radical from 2,4'- or 4,4'-DDD, and CCl₃ from 2,4'- or 4,4'- DDE then leads to the formation of a stable benzylic carbocation. Daughter ions with mass/charge (m/z) values of 200 and 165 are formed respectively as a result of the loss of one or two more chlorines (Chusaksri et al., 2006).

In this study, a new HPLC-UV method that measures DDT and DDE levels in human blood and modified RPMI media used for malaria culturing is described. Analyses were performed on spiked biological samples to determine the recovery efficiency and analytical sensitivity of the method. Procedures are described for the simultaneous determination of trace levels of DDT and DDE in biological media using a comparison of three analytical methods, GC-MS in split mode, GC-MS in splitless mode, and HPLC-UV.

p,p'-DDT and p,p'-DDE were selected for analysis since p,p'-DDT is the isomeric form of technical DDT that has the greatest relative abundance of 77.1 %, and p,p'-DDE is its principle metabolite (Smith, 1991; WHO, 1979). The procedure consists of a solvent extraction step (Liu
and Pleil 2002). Determination was carried out using two GC-MS methods based on that of Al-Saleh et al., 2002 and a novel HPLC-UV method developed for this study. These chromatographic methods were developed taking into account the criteria established for the validation of routine quantitative analytical compounds (Dobson et al., 1990; Shah et al., 1992; Van Pelt et al., 1998).

The limitations and advantages of using GC-MS or HPLC-UV techniques that avoid the use of clean-up steps for quantitative and qualitative analyses of blood serum and RPMI media samples are compared and discussed. This work mainly focuses on obtaining a convenient method for the simultaneous determination of the DDT and DDE in serum/blood/media using LLE-GC-MS and LLE-HPLC-UV methods. GC-MS and HPLC-UV methods were evaluated with respect to the analytical figures of merit required for routine analysis, (Shah et al., 1992; Dobson et al., 1990; Bennett et al., 1997) such as linearity, limits of detection (LOD), quantification limits (LOQ), quality control (QC) standards, and recovery experiments.

2.2 Methods

2.2.1 Sample collection
As a pilot study, samples of left over anonymous blood donated by the Western Cape blood bank in South Africa, or alternatively, blood from the researcher conducting this study were used for analysis. No ethical clearance was therefore required to allow use of these blood samples for analysis.

The blood was drawn into 4.5 mL glass vacutainer tubes containing sodium citrate (Na-citrate) to prevent blood clotting. Extracted samples were stored at 4-8 ºC until required for analysis, at most for 5 days.

2.2.2 Drying and storage of reagents and products
All chemicals and reagents used in this study including \( p,p' \)-DDT, \( p,p' \)-DDE were purchased from Sigma-Aldrich (Steinheim, Germany) unless otherwise stated. These reagents together with evaporated extracts prepared from the extraction processes as described in Sections 2.2.5 and 2.2.6 were stored at room temperature except for RPMI 1640, albumax II and gentamycin that were stored at 4 ºC.
To avoid detergent contamination of the samples through the glassware, after the regular wash, the glassware was then rinsed three times with distilled water, three times with 60% ethanol, then another three times with analytical grade water (Rautenbach, 1999).

A Millipore Milli Q® water system (Milford, USA) was used to prepare the analytical grade water by filtering it from a reverse osmosis plant. The glassware was then placed in an oven to dry at temperatures ranging between 110ºC and 140ºC, and then pyrolised at 565-570ºC for 45 minutes to ensure organic-free and clean glassware (Rautenbach, 1999).

2.2.3 Analytical weighing of \( p,p' \)-DDT and \( p,p' \)-DDE

Using an analytical weighing procedure developed in our laboratory, a six digit analytical weighing balance was used to determine the weights of \( p,p' \)-DDT and \( p,p' \)-DDE at very high levels of precision and accuracy.

The glass vials, cleaned as described in Section 2.2.2, were first labelled using a diamond-tip glass marker. The vials were then dried in an oven at 120 ºC for 20 minutes to remove moisture before being placed in a vacuum desiccator for another 20 minutes where the vials were left to cool.

For weighing, the vials were transferred directly into the six digit analytical weighing balance chamber which was kept dry by placing glass vials with phosphorous pentoxide (Sigma-Aldrich) as a drying agent into the chamber beforehand. Also, tongs were used to handle the vials to be weighed instead of bare fingers as finger prints would also affect the analytical determination of the vial and sample weights.

The process of drying the vials in the oven for 20 minutes, putting them in the desiccator for another 20 minutes and then checking the weight of the vials was repeated at least three times and the weights were recorded to six decimal places. Vial weights were then averaged to establish the final vial weight.

\( p,p' \)-DDT and \( p,p' \)-DDE were each weighed separately by placing the compounds into the vials and taking at least three separate measurements of the vials and their contents within 0.000050 g error. The masses were averaged and the final mass of the vials and their contents established. The analytically weighed compounds in the vials were then labelled and capped, then sealed with
parafilm around the neck of the cap and placed in a desiccator at room temperature until required for use.

2.2.4 Preparation of solutions of \( \text{p,p}'-\text{DDT} \) and \( \text{p,p}'-\text{DDE} \)

Hamilton glass pipettes and pyrolised glass vials were used throughout solution preparation of \( \text{p,p}'-\text{DDT} \) and \( \text{p,p}'-\text{DDE} \) to prevent loss of the compounds through use of plastic tips and tubes due to the hydrophobic nature of the compounds.

Initial spiked solutions and standard solutions of each of the compounds were prepared by dissolving the respective analytically weighed samples of each of \( \text{p,p}'-\text{DDT} \) and \( \text{p,p}'-\text{DDE} \) as described in Section 2.2.3, first in an appropriate volume of HPLC grade methanol (Romil Ltd, Cambridge, UK) to obtain 5.00 mg/mL stock solutions, then in GC grade dichloromethane (Sigma-Aldrich) to the specific concentrations of 5.00 and 10.0 µg/mL (parts per million or ppm) for GC-MS analysis. For HPLC analysis, all samples were prepared in HPLC grade methanol regardless of their concentrations.

2.2.5 Extraction and analysis of \( \text{p,p}'-\text{DDT} \) and \( \text{p,p}'-\text{DDE} \) spiked in human blood and RPMI media

The extraction protocol used in this study was based on a slight modification of that used by Liu and Pleil (2002). All samples were prepared and run at least in triplicate unless otherwise stated. Hexane (Merck, RSA) or hexane:ethyl acetate (BDH Chemicals Ltd, Poole, England) (4:1, \( v/v \)) extracting solvents were used as extraction blank controls. Unextracted \( \text{p,p}'-\text{DDT} \) and \( \text{p,p}'-\text{DDE} \) spiked solvents were used as the extraction positive controls in which percentage recovery of the \( \text{p,p}'-\text{DDT} \) and/or \( \text{p,p}'-\text{DDE} \) in the solvent would be assumed to be 100 % since none of the compounds are lost.

Aldrin (200 µg/mL in iso-octane) used as internal standard at a concentration of 10 ppm was added to all samples analysed by the GC-MS method 2, described in Section 2.2.8. Aliquots (4.50 or 9.00 µL) of the 5.00 mg/mL stock solutions of \( \text{p,p}'-\text{DDT} \) and/or \( \text{p,p}'-\text{DDE} \) prepared as described in Section 2.2.4 were mixed with 1.50 mL human blood or RPMI media followed by the addition of 1.5 mL MilliQ water to achieve spiked sample concentrations of 5 ppm or 10 ppm, respectively, in a 4.50 mL extraction solvent volume.
Sodium chloride (0.500 g NaCl) was then added to each sample to saturate the blood or media solutions. The NaCl also lead to the lysis of the erythrocytes in the blood samples. The resulting solutions were extracted with 3.00 mL extraction solvent by vortexing for 5 minutes to ensure extraction of the compounds from the biological media into the organic solvent. An Eppendorf Centrifuge 5702 with a swing-bucket rotor type A-8-17 at 1600 x g for 30 minutes was used to aid the separation of layers into the biological media (blood, RPMI media) layer and the organic solvent layer. After separation of the organic phase (top layer, organic solvent with DDT and/or DDE) from the aqueous phase (bottom layer, biological medium), the aqueous phase was then extracted again using the same procedure as described above with 1.50 mL of extraction solvent. The organic extracts were then combined and dried by the addition of 0.200 g anhydrous sodium sulphate (Merck Ltd, UK) such that sodium sulphate crystals no longer clumped together, to get rid of any traces of water.

Before adding the anhydrous sodium sulphate to the combined extracts, empty test tubes were weighed and reweighed after the addition of the combined organic extracts. This was done to calculate the volume of extraction solvent that was retained after the extraction procedure as there may have been some solvent losses during the extraction procedure (1.00 mL hexane: ethyl acetate 4:1 v/v ~ 0.70 g). This would therefore assist in accounting for volumetric or \( p,p' \)-DDT and/or \( p,p' \)-DDE recovery errors.

The anhydrous extracts were then transferred into clean glass vials upon which these solutions were then evaporated under \( \text{N}_2 \) flow to total dryness until required for analysis. The dried residues (evaporated hexane or hexane: ethyl acetate (4:1, v/v) extracts) were kept at room temperature until required for analysis. To concentrate the samples so that the compounds \( p,p' \)-DDT and \( p,p' \)-DDE could be easily visualised and identified, the dried extracts were then reconstituted in only 450.0 or 900.0 µL GC grade dichloromethane or HPLC grade methanol on the day of the GC-MS or HPLC analysis, respectively, to achieve analysis concentrations of for example 50 and/or 25 ppm, respectively, in the spiked samples. Where possible, samples were analysed the day after the extraction to prevent losses of the compounds in the biological media due to long periods of storage. However, remaining extract samples already reconstituted in solvent that were not analysed were stored in tightly sealed vials at 4°C.
2.2.6 Extraction and analysis of DDT and DDE in unspiked human blood and RPMI media

To determine the DDT and/or DDE contaminant variables in biological media samples, background levels of the compounds in the various blood (packed erythrocytes or whole blood) and malaria culturing RPMI media were assessed.

Extraction of the compounds was carried out in a similar fashion as described in Section 2.2.5, except that the samples were not spiked with solutions of \( p,p' \)-DDT or \( p,p' \)-DDE. Each extraction and analysis was done at least in triplicate unless otherwise stated. The blank experiment control, 1.500 mL analytical grade water was extracted using the same method as above.

2.2.7 HPLC analysis

The extracts were prepared using the exact sample extraction and preparation protocols described in Sections 2.2.5 and 2.2.6. The reconstitution solvent was 90 % HPLC grade methanol which was also used to prepare the standard solutions of \( p,p' \)-DDT and \( p,p' \)-DDE, as well as to reconstitute the evaporated extracts before HPLC analysis.

Based on the HPLC method developed in our laboratory as described below, a 50.0 µL aliquot of all blank (unspiked) samples was injected into the HPLC system so as to be able to identify any trace amounts of DDT and/or DDE in the blank samples if present. Meanwhile, an injection volume of 10.0 µL was used for all \( p,p' \)-DDT and \( p,p' \)-DDE spiked samples as well as standard solutions.

Reverse phase HPLC was used to analyse and quantify the samples. A Nova-Pak\textsuperscript{®} C\textsubscript{18} HPLC column (5 µm particle size, 60 Å pore size 150 mm x 3.9 mm) was used with a chromatography system consisting of two Waters 510 pumps, Millennium\textsuperscript{32} software control system, Waters Model 440 detector and a WISP 712 sample processor. The chromatographic separation was monitored at 254 nm using a Waters Model 440 UV-detector. Linear and non-linear gradients were created using eluant A (0.1 % triflouroacetic acid (TFA) in MilliQ water) and eluant B (90 % acetonitrile (ACN) from Romil Ltd and 10 % eluant A).

Eluants A and B were filtered using 0.45 µm HLPV filters to remove any residual particulate thus protecting the HPLC system and column from blockage by dirt particles. Eluants were
degassed by sonication for 20 minutes prior to use. The optimised HPLC method used in this study is described in Table 2.1 at a flow rate of 1.0 mL/min. The gradient program was specifically developed for the separation of \( p,p'\)-DDT and \( p,p'\)-DDE, as well as separation of the two compounds from compounds in the biological media that absorb light at 254 nm. Acquired data was processed and reviewed using the Millennium\textsuperscript{32} software package.

Table 2.1 Optimised gradient program used for the chromatography of \( p,p'\)-DDT and \( p,p'\)-DDE.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow rate (mL/min)</th>
<th>% A</th>
<th>% B</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.0</td>
<td>40</td>
<td>60</td>
<td>Initial gradient</td>
</tr>
<tr>
<td>4</td>
<td>1.0</td>
<td>0</td>
<td>100</td>
<td>Isocratic separation of DDT, DDE</td>
</tr>
<tr>
<td>14</td>
<td>1.0</td>
<td>0</td>
<td>100</td>
<td>Regeneration and equilibration</td>
</tr>
</tbody>
</table>

2.2.8 GC-MS method 1

The first GC-MS analysis was used in split mode to confirm the identity of the eluting compounds. Solvent extracts were analysed for levels of \( p,p'\)-DDT and \( p,p'\)-DDE using a Waters GCT Premier instrument with an HP5 column of 30 m length, 0.25 mm internal diameter and 0.25 µm film thickness. The GC-MS method used was based on the method described by Al-Saleh \textit{et al.} (2002). The mass spectrometer was operated in electron-impact (EI) ionization mode with electron impact energy of 70eV. The injector temperature was 250 °C and the transfer line was kept at 250 °C. Helium, the carrier gas, had a flow rate of 1 mL/min. The split ratio used was 1:5.

The oven temperature ramps involved initially maintaining the column oven temperature at 80 °C for 1.2 minutes after injection, then programming it at 30 °C/min to 170 °C and holding for 4 minutes before moving to 225 °C at 2.5 °C/min, holding for another 3 minutes at 275 °C at 30 °C/min before reaching 300 °C at 30 °C/min in the final ramp.

The samples were analysed using a full scanning mass range of 50 to 450 \( m/z \) (perfluorotri-N-butylamine as mass reference) through which spectra were attained and background compounds found. Quantification was performed using selected ion monitoring (SIM) mode in which
individual ions for each of the selected compounds, \( p, p' \)-DDT and \( p, p' \)-DDE were monitored. MassLynx V4.0 (Micromass Ltd.) software was used for data analysis.

### 2.2.9 GC-MS method 2

A second GC-MS analysis method (GC-MS2) in splitless mode was used to improve the limit of detection (LOD) and limit of quantification (LOQ) values so as to be able to detect low levels of \( p, p' \)-DDE and \( p, p' \)-DDT by optimising the previously used method described in Section 2.2.8. The sample run time was reduced to 20 minutes and samples with a much lower \( p, p' \)-DDE and \( p, p' \)-DDT concentration, \( \leq 10 \) ppm (parts per million), were analysed.

Modifications were made to the initial GC-MS method 1 described in Section 2.2.8. A Waters GCT equipped with CTC CombiPAL autosampler, and an HP5 column similar to that used for the GC-MS1 analysis was used. The injector temperature was changed to 300 °C. A 2 µL injection volume was used in splitless mode. The oven temperature programme involved holding the column for 5 minutes at an initial temperature of 100 °C. The oven temperature ramps then changed to 170 °C at 30 °C/min. The temperature was held at 170 °C for 4 minutes before going up to the final temperature of 320 °C at 30 °C/min, where again the temperature was held for 5 minutes. The scan time was 0.15 minutes with an inter-scan delay of 0.05 min.

A 10 ppm spike of aldrin solution (200 µg/mL in iso-octane) was used as internal standard in all samples prepared for GC-MS method 2 analyses to compensate for the injection error and improve repeatability of results.

### 2.2.10 Recovery

This set of experiments was done to establish the extraction efficiency of the compounds from the biological media. Recovery of \( p, p' \)-DDT and \( p, p' \)-DDE from the blood and media samples was determined by dividing the blood and media samples into three equal portions of 1.500 mL each in which one of the three samples was unspiked (blank) and the other two portions were spiked with standard solutions of 5.00 ppm or 10.0 ppm each \( p, p' \)-DDT and/or \( p, p' \)-DDE.

One blank solvent sample and two solvent samples spiked with \( p, p' \)-DDT and \( p, p' \)-DDE each in the same concentrations as in blood and media, were used to determine the theoretical 100 % recovery. These solvent samples were not extracted (positive controls), thereby allowing the
assumption of full recovery of the \( p,p' \)-DDT and \( p,p' \)-DDE being 100 % in the spiked solvent. 4.50 mL solvent was spiked with 4.50 µL or 9.00 µL of the 5mg/mL stock solutions of \( p,p' \)-DDT and/or \( p,p' \)-DDE to achieve concentrations of 1, 5 and 10 ppm respectively. The solution was dried by a stream of N\(_2\) flow then reconstituted in 4.50, 0.450 or 0.900 mL dichloromethane or 90 % HPLC grade methanol, and analysed using the same GC-MS and HPLC-UV methodologies described above in Sections 2.2.7, 2.2.8 and 2.2.9, respectively.

The percentage recovery of the compounds in the biological media was determined by using the peak area (PA) obtained for the \( p,p' \)-DDT and/or \( p,p' \)-DDE spiked in blood and RPMI media in relation to the peak areas of \( p,p' \)-DDT and/or \( p,p' \)-DDE in solvent, respectively, and was calculated using the equation below. DDx represents \( p,p' \)-DDT or \( p,p' \)-DDE.

Equation 2.1: 

\[
\% \text{ Recovery} = \left( \frac{P_{\text{DDx in blood/media}}}{P_{\text{DDx in 500 µL solvent}}} \right) \times 100
\]

2.2.11 Standard curves

Standard curves of doubling dilution series of 100 ppm to 7.8 ppb for HPLC-UV and 10 ppm to 78 ppt for GC-MS were set up to assist in quantification of the data. Standard solutions of \( p,p' \)-DDT and \( p,p' \)-DDE were serially diluted in GC grade dichloromethane or HPLC grade methanol depending on the method of analysis.

Each of the standard solutions was prepared in triplicate from three different separately prepared stock solutions for the two GC-MS and one HPLC-UV analyses. For HPLC-UV analysis, the standard solutions were prepared in 90% HPLC grade methanol. GC grade dichloromethane was used to prepare standard solutions for the GC-MS analyses.

The peak area of each eluted compound was determined and correlated with the different \( p,p' \)-DDT and \( p,p' \)-DDE concentrations in µg/mL (ppm) injected on the GC-MS and HPLC systems. To analyse the relationship between the compound concentrations and peak areas, linear regression analysis was done using Prism 4 (GraphPad Software, San Diego, CA, USA). The parts of the standard curve that adhere to 95% confidence intervals as well as residual errors
within 10% error were used to derive the linear equation for calculation of sample concentrations.

The limit of detection (LOD) was defined as the lowest standard concentration within the range on the standard curve, of \( p,p' \)-DDT and \( p,p' \)-DDE where the compounds could still be detected above the background. The limit of quantification (LOQ) was defined as the lowest standard concentration on the calibration curve at which the compound concentrations were most reliably obtained within the linear section and 95 % confidence interval, as well as within 10% residual error.

Spiking of the solvent was repeated each time spiked blood or media samples were extracted to make certain of repeatability of results, and for statistical purposes.

2.3 Results and Discussion

2.3.1.1 HPLC separation of \( p,p' \)-DDT and \( p,p' \)-DDE

In the initial development of the HPLC-UV method, several elution gradients and solvent compositions were attempted to try and separate \( p,p' \)-DDT and \( p,p' \)-DDE. First, an isocratic mode was used to separate the compounds with 90% ACN flowing at 1.00 mL/minute. \( p,p' \)-DDT and \( p,p' \)-DDE both eluted too early (1.95 and 2.18 minutes) with the polar compounds absorbing at 254 nm (results not shown).

The mobile phase was then changed by addition of TFA and using different ratios of eluants A (0.1% TFA in MilliQ water) and B (90% ACN + 10% eluant A). We started with 50 % each of both solvents from 0-3 minutes. The solvent composition then changed to 25 % A and 75 % B from 3 to 10 minutes and eluant A was increased to 100 % between 10 to 14 minutes. \( p,p' \)-DDT and \( p,p' \)-DDE were resolved and eluted after roughly 7 and 9 minutes respectively (results not shown). However the peaks were broad and they co-eluted with other unidentified peaks of possibly polar compounds, at the respective retention time regions.

A third attempt was made by starting with 50:50 eluants A:B solvent ratio and then the running a linear gradient from 4-10 minutes to 75% eluant B, followed by a gradient to 100 % B between 10 and 14 minutes. This method produced a good separation of the compounds with narrow,
sharp peaks. \( p,p' \)-DDT and \( p,p' \)-DDE eluted at 10.1 and 10.5 minutes, respectively (results not shown). The peaks, however, eluted very closely and also co-eluted with contaminant peaks.

The final attempt in the method optimisation entailed running the gradient from 40 to 100% eluant B from 4 to 14 minutes. This method also produced a good separation of the compounds with narrow, sharp peaks. Chromatographic HPLC separation of \( p,p' \)-DDT and \( p,p' \)-DDE are shown in Figure 2.2, with retention times 9.4 and 9.8 minutes, respectively, over a 20 minute chromatographic run (Figure 2.2).

![Figure 2.2 Typical HPLC chromatograms of standard solution of \( p,p' \)-DDT and \( p,p' \)-DDE (90:10 ppm mixture), blood and blood spiked with 5 ppm \( p,p' \)-DDT, respectively. The chromatographic method is described in Section 2.2.7.](attachment:image.png)
Both \( p,p' \)-DDT and \( p,p' \)-DDE were completely resolved from each other and UV visible contaminants using the optimised method. Based on the elution profile, most polar contaminating compounds eluted in the first few minutes of the run whilst the more hydrophobic contaminants eluted well after the target compounds \( p,p' \)-DDT and \( p,p' \)-DDE.

No apparent interfering or co-eluting peaks with similar retention times were found in the chromatograms of blank water and solvent samples although some interference or residual \( p,p' \)-DDT and \( p,p' \)-DDE were evident in blood, RPMI media and 2% haematocrit cultures.

The described cost effective optimised HPLC-UV method (Section 2.2.7, Table 2.1) with liquid-liquid extraction that was developed as an alternative to the much more expensive GC-MS method proved to be efficient for simultaneously separating and quantifying \( p,p' \)-DDT and \( p,p' \)-DDE without an extra purification step.

### 2.3.1.2 GC-MS separation and identification of \( p,p' \)-DDT and \( p,p' \)-DDE

Combined with liquid-liquid extraction, a GC-MS method was necessary for the identification during separation and quantification of \( p,p' \)-DDT and \( p,p' \)-DDE. The selected-ion monitoring (SIM) for \( p,p' \)-DDT and \( p,p' \)-DDE respectively, as shown in Table 2.2, was used to carry out quantitative analyses and confirmation of the compounds’ identities at the specific retention time regions as determined by the chromatography of the DDT and DDE standards. Confirmation of the compounds’ identities using the GC-MS methods was based on the detection of the molecular ion, two or three distinct fragment ions, as well as matching the chromatographic retention time to that of the standard for each analyte. A GC-MS library search was also used to confirm the identity of the compounds by comparing the obtained fragmentation profiles of the compounds to those in the library.

*Figure 2.3* shows the fragmentation patterns of \( p,p' \)-DDT and \( p,p' \)-DDE standard solutions, in which the selected ions for the GC-MS methods are indicated with arrows. The standard solutions of the analytes were prepared as described in *Section 2.2.4*. The two ions used for the detection of \( p,p' \)-DDT were 235 and 237, whilst the three ions used for \( p,p' \)-DDE detection were 246, 248 and 318 for both GC-MS methods, respectively.
The original GC-MS method (GC-MS method 1) was run over 35 minutes using a 5:1 split (0.2 of 1.0 µL injected on column) to limit column contamination. Separation was obtained with the retention times at 20.74 minutes and 25.70 minutes for \( p,p' \)-DDE and \( p,p' \)-DDT, respectively \((Figure\ 2.4)\). However, we observed that blood spiked with 5 ppm \( p,p' \)-DDT showed degradation of the compound into its main metabolites, DDD and DDE. A similar pattern was observed for the standard samples that were not extracted \((Figure\ 2.4)\).

\( Figure\ 2.3\) Fragmentation patterns for \( p,p' \)-DDT and \( p,p' \)-DDE. MS was used to monitor the quantification ions with \( m/z \) 235 and 237 for \( p,p' \)-DDT and \( m/z \) 246, 248 and 318 for \( p,p' \)-DDE as shown by the fragmentation patterns.
Table 2.2 \( p,p' \)-DDT and \( p,p' \)-DDE identification in blood and RPMI media studied with GC-MS as shown by the optimized chromatograms in Figure 2.3.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>( t_R ) (min)</th>
<th>Monitored ions (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( p,p' )-DDT</td>
<td>25.70</td>
<td>235 + 237</td>
</tr>
<tr>
<td>( p,p' )-DDE</td>
<td>20.74</td>
<td>246 + 248 + 318</td>
</tr>
</tbody>
</table>

\( t_R \) = retention time in minutes

The DDT breakdown was an indication of the harshness of the GC-MS process and was an unwanted process as DDT must remain intact for identification and quantification. The more stable \( p,p' \)-DDE, however, remained intact under the same conditions.

The chromatogram (Figure 2.4) of unspiked blood showed a small peak at the retention time for \( p,p' \)-DDE which was expected to be identified as DDE, since the blood used for this analysis was donated by an individual who may have been at some point in their life exposed to DDT either as part of malaria control or in its use as a pesticide in agriculture or both. The peak was, however, identified as a long hydrocarbon chain although there was some doubt as to this conclusion since GC-MS method 1 had not been optimized to detect low levels of the compounds in the biological media. The levels in the blood may have been lower than the limit of detection (LOD).

GC analysis requires the optimization of injection conditions which is essential in method development (Tuinstra et al., 1985; Lang, 1992). Although on-column injection has also been used, most GC applications for PCB and OCP analysis have employed split/splitless injection systems. In as much as on-column injection avoids artefacts associated with heated split/splitless systems (that is, degradation of labile compounds), it requires pure extracts to avoid matrix effects (Lang, 1992).

In the “split mode” injection in our GC-MS method 1 analysis, only 0.2 µL of the original 1.0 µL injection is introduced onto the column. This permits the analysis of complex samples, because it limits the contamination on the column. However, this volume was deemed insufficient to enable accurate determination of either DDT and/or DDE in the samples.
Figure 2.4 GC-MS method 1 chromatograms of \( p,p' \)-DDT and \( p,p' \)-DDE standards, blood (packed erythrocytes), 5 ppm \( p,p' \)-DDT spiked blood and 5 ppm \( p,p' \)-DDE spiked blood. The chromatograms were obtained using GC-MS method 1 described in Section 2.2.8.
In the GC-MS method 2 a higher injection volume of 2.0 µL in split less mode was used, as our three times liquid extraction of samples limited the contamination of the column. The chromatographic run in the GC-MS method 2 was optimised to be much shorter with \( p,p' \)-DDE and \( p,p' \)-DDT eluting at 13.65 minutes and 14.30 minutes respectively (Figure 2.5). Aldrin eluted at 12.65 minutes. We were unable to overcome or limit the DDT degradation in the optimised GC-MS method. This was not the case with the HPLC-UV method in which DDT remained intact as shown in Figure 2.2.

Figure 2.5 GC-MS method 2 chromatograms of a standard mixture of 5 ppm \( p,p' \)-DDT and \( p,p' \)-DDE, RPMI medium spiked with 5 ppm \( p,p' \)-DDT and \( p,p' \)-DDE and unspiked RPMI medium. The chromatograms were obtained using optimized conditions described in Section 2.2.9.

The DDE and DDD peaks in the unextracted \( p,p' \)-DDT standard samples (Figure 2.5) were, however, \( \pm 36\% \) smaller than those in the extracted spiked modified RPMI media used for malaria culturing. The biological RPMI media showed background levels of DDE which were added to the spiked concentration and thereby leading to a slightly higher concentration being
detected during GC-MS analysis (Figure 2.5). The complex media could also have protected DDT from degrading as observed for the standards in the GC-MS.

Although background levels of a variety of non-target compounds were found when hexane-ethyl acetate (4:1, v/v) was used as the extraction solvent, this solvent combination was more aggressive than hexane, yielding higher recoveries of the compounds as further described in Section 2.3.2.

2.3.2 Recovery efficiency study

The extraction efficiency of \( p,p' \)-DDT and \( p,p' \)-DDE from human blood and malaria culturing RPMI media was assessed with our analytical methods in a recovery efficiency study in which the biological samples were spiked with specific amounts of \( p,p' \)-DDT and \( p,p' \)-DDE. Initially the recovery of DDT and DDE was <50% because of the use of plastic pipette tips in the volume measurement and transfer of samples. When the use of plastic was eliminated from all the steps in preparation and extraction the recovery yields from most of the media were consistently above 90%. The peak area of each analyte, using the two GC-MS methods and the HPLC-UV method, was compared to a standard curve. The percentage recovery was calculated based on Equation 2.1 and summarised in Table 2.3 for the two GC-MS methods and HPLC-UV method.

Table 2.3 Absolute recoveries of \( p,p' \)-DDT and \( p,p' \)-DDE in blood and RPMI media by GC-MS and HPLC-UV analyses (n represents number of repeats).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Spiked Sample</th>
<th>% Recovery±SD GC-MS Method 1 (n)</th>
<th>% Recovery±SD GC-MS Method 2 (n)</th>
<th>% Recovery±SD HPLC-UV (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( p,p' )-DDT</td>
<td>Blood</td>
<td>99.99 ± 11.6 (6)</td>
<td>nd</td>
<td>91.9 ± 6.50 (8)</td>
</tr>
<tr>
<td></td>
<td>RPMI media</td>
<td>86.6 ± 3.9 (2)</td>
<td>nd</td>
<td>103 ± 7.53 (7)</td>
</tr>
<tr>
<td></td>
<td>2% haematocrit in media</td>
<td>nd</td>
<td>nd</td>
<td>104 ± 5.67 (7)</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>92.4 ± 14.7 (4)</td>
<td>nd</td>
<td>106 ± 10.9 (7)</td>
</tr>
<tr>
<td>( p,p' )-DDE</td>
<td>Blood</td>
<td>89.1 ± 14.6 (6)</td>
<td>113 ± 12.3 (10)</td>
<td>88.2 ± 8.00 (8)</td>
</tr>
<tr>
<td></td>
<td>RPMI media</td>
<td>109 ± 17.7 (2)</td>
<td>114 ± 4.88 (5)</td>
<td>96.1 ± 5.00 (7)</td>
</tr>
<tr>
<td></td>
<td>2% haematocrit in media</td>
<td>nd</td>
<td>113 ± 10.9 (5)</td>
<td>93.5 ± 6.52 (7)</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>91.0 ± 18.7 (4)</td>
<td>100.0 ± 7.18 (5)</td>
<td>96.9 ± 4.92 (8)</td>
</tr>
</tbody>
</table>
The first attempts to extract \( p,p'\)-DDT and \( p,p'\)-DDE from blood and media samples involved using hexane as the extraction solvent. The extraction efficiencies using hexane or hexane: ethyl acetate (4:1, \( v/v \)) as extraction solvents showed that the latter yielded the best recovery of \( p,p'\)-DDT and \( p,p'\)-DDE from the biological media. Findings from Liu and Pleil (2002) also showed that the recoveries from hexane: ethyl acetate (4:1, \( v/v \)) extractions were better than the hexane extractions. Plasticisers in the form of phthalates were identified with GS-MS as a major group of interfering compounds in media/blood containing Albumax\textsuperscript{TM}, probably originating from the preparation itself or from the caps of the blood vacutainer tubes, as these were the only source of plastic available. To minimise these interferences, all glassware was pyrolysed before use as described in Section 2.2.2.

According to the HPLC analysis of the different extracted samples the absolute recovery for \( p,p'\)-DDT was between 92 and 106\% and that of \( p,p'\)-DDE between 88 and 97\% (Table 2.3). This improved on the DDT and DDE recovery of 60 to 85\% and 67 to 99\%, respectively, reported in literature (De Francia \textit{et al.}, 2006). The recovery from blood samples was improved from 82\% to 94\% for \( p,p'\)-DDT and from 82\% to 91\% for \( p,p'\)-DDE when the blood was extracted three times. This extra extraction step for \( p,p'\)-DDT and \( p,p'\)-DDE was deemed necessary because DDT and DDE could interact with the lipids in blood and the Albumax\textsuperscript{TM} containing bovine albumin in the malaria culturing media.

For assessing recovery with GC-MS, RPMI media adapted for growing malaria cultures, was spiked with 5 ppm of \( p,p'\)-DDT and \( p,p'\)-DDE and extracted with hexane: ethyl acetate (4:1, \( v/v \)) before being analysed. The influence of Albumax\textsuperscript{TM} (a serum albumin substitute) and RPMI alone was also assessed. A higher percentage recovery than expected (109 ± 17.7 \%) of \( p,p'\)-DDE from the RPMI media was found indicating the possibility of residual \( p,p'\)-DDE in one of the media components. \( p,p'\)-DDT spiked RPMI media gave slightly lower recoveries of 86.6 ± 3.9 \% indicating possible interaction of \( p,p'\)-DDT with the lipid rich Albumax\textsuperscript{TM} thus making its extraction from the media more difficult. Since Albumax\textsuperscript{TM}, a constituent of modified RPMI media, is extracted from bovine serum in cows from New Zealand where DDT pesticide agriculture was practiced until ban of the insecticide in the country (Shivaramaiah \textit{et al.}, 2002), there was a very strong possibility of background levels of DDT and/or DDE in the media.
The GC-MS analyses of the different extracted samples showed absolute recovery for \( p,p' \)-DDT between 87 and 100% and that of \( p,p' \)-DDE between 91 and 113% (Table 2.3). This correlated well with \( p,p' \)-DDT and \( p,p' \)-DDE recoveries of 67.8 to 106.6% and 98.3 to 110.0%, respectively, reported in literature (Liu and Pliel 2002; Al-Saleh et al., 2005). The second GC-MS method showed improved variations (lower standard deviation, <10%, and % coefficient of variation (% CV) of 4 to 10%) in the percentage recoveries of \( p,p' \)-DDE from the biological samples except for in blood where the %CV was 11%. The \( p,p' \)-DDE percentage recovery itself increased from 89 % to 113 % in the GC-MS method 2 analysis possibly because of the varying levels of DDT and/or DDE found in the blood donated from the blood bank from the different donor batches received each week.

The recovery of \( p,p' \)-DDT was consistently higher with a smaller error (<10 % CV) as detected by HPLC (except for \( p,p' \)-DDT from water) compared to GC-MS method 1 analysis (Table 2.3). This could be due to the breakdown of DDT in the GC-MS and/or interfering compounds in the HPLC analyses. Alternatively, the 12-16% higher recovery from the media and blood than the recovery from water, as detected with HPLC, could be due to residual \( p,p' \)-DDT. The observed \( p,p' \)-DDE recoveries were generally higher using GC-MS method 2 analysis compared to HPLC analysis as shown in Table 2.3. This lower detection using HPLC could be due to slight loss of detector linearity at higher \( p,p' \)-DDE concentrations. Alternatively, the 13-14% higher recovery from the media and blood than the recovery from water, as detected with the more sensitive GC-MS method 2, could be due to residual \( p,p' \)-DDE in samples. These, however, are slight variations which may have been due to volumetric errors encountered during the LLE step described in Section 2.2.5.

2.3.3 Evaluation of HPLC-UV and GC-MS methods for DDT and DDE quantification

GC-MS and HPLC-UV derived standard curves for \( p,p' \)-DDT and \( p,p' \)-DDE were set up for the quantitative determination of the compounds in the biological media as described in Section 2.2.11.

2.3.3.1 HPLC-UV standard curves for DDT and DDE quantification

The peak areas of \( p,p' \)-DDT and/or \( p,p' \)-DDE obtained with our optimised HPLC-UV method showed for a standard solution of equal concentrations of \( p,p' \)-DDT and \( p,p' \)-DDE, that \( p,p' \)-
DDE had a much greater absorption intensity at 254 nm which almost overshadowed the peak for \( p,p'-\text{DDT} \). This indicated that our sensitivity for \( p,p'-\text{DDE} \) is expected to be higher than that of \( p,p'-\text{DDT} \). We therefore adjusted the standard curve ranges accordingly. Linear calibration curves for \( p,p'-\text{DDT} \) and \( p,p'-\text{DDE} \) were obtained over the concentration range 78 ppb to 100 ppm for \( p,p'-\text{DDT} \) and 7.8 ppb to 50 ppm for \( p,p'-\text{DDE} \) (Figure 2.6 and Table 2.4). These standard solutions were prepared from three different stock solutions each for \( p,p'-\text{DDT} \) and \( p,p'-\text{DDE} \). The correlation coefficients (\( R^2 \)) for the linear calibration curves were \( \geq 0.999 \), thus giving repeatable best fit lines within 95 % confidence interval without use of an internal standard. The residuals for these fits over the whole range, using unweighted regression analysis, showed between 0.4% and 9.2% residual error for up to 1.563 ppm, but only 2-6% CV over the whole concentration ranges for both \( p,p'-\text{DDT} \) and \( p,p'-\text{DDE} \). The biggest error in terms of the 95 % confidence interval was observed below 1.563 ppm for both \( p,p'-\text{DDT} \) and \( p,p'-\text{DDE} \) (Figure 2.5 A, B and D) if a linear fit over the whole concentration range was considered. Using unweighted regression analysis the LOQ for both compounds was 1.563 ppm. However, we found bias towards the high concentrations in these unweighted linear curves and could improve the LOQ by approximately 200 times to 7.8 ppb for DDE by fitting \( 1/Y^2 \) weighted regression line (Figure. 2.5 B and D). Similarly, the LOQ improved by approximately 20 times to 78 ppb for DDT by weighted fitting (Figure. 2.5 B and C). The limit of detection of \( p,p'-\text{DDT} \) and \( p,p'-\text{DDE} \) in pure samples was found to be 78 ppb and 7.8 ppb respectively. The repeatability of the HPLC analysis of DDT and DDE was very good and calibration curves from one set of analyses to the next remained with the average %CV for DDE at 2.4% and that of DDT at 6.4% over three standard curve repeats (results not shown). The summary for the regression analysis is given in Table 2.4 and the graphical representations are given in Figure 2.6.
Figure 2.6 Standard curves for \( p,p' \)-DDT and \( p,p' \)-DDE obtained with the HPLC-UV method. (A) shows the linear regression line though the triplicate repeats of the \( p,p' \)-DDT and \( p,p' \)-DDE calibration curves. In graph B the \( y \) axes is converted to \( \log_{10} \) scale to show the error at lower concentrations, as depicted by 95% confidence interval (vertical lines) at the lower concentrations. The linear regression line in graphs were fitted with (B) and without (A, B) weighing with \( 1/Y^2 \) to minimise relative square distances. In graphs C and D the prediction of accuracy using the regression line equations (B) is depicted respectively for the \( p,p' \)-DDT and \( p,p' \)-DDE calibration curves. All standard curves were analysed as described in Section 2.2.7. Mean ± SEM for three determinations is shown for each data point in B, C and D.
Table 2.4 Summary of data calculated for fitting of standard curves for \( p,p' \)-DDT and \( p,p' \)-DDE from HPLC-UV analysis of standard concentration ranges (described in Section 2.3.3.1).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Linear regression equations from fits to standard conc. ranges</th>
<th>( R^2 )</th>
<th>LOQ</th>
<th>LOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>( p,p' )-DDT</td>
<td>( y = 1446x + 449.8 )</td>
<td>0.9997</td>
<td>1.56 ppm</td>
<td>78 ppb</td>
</tr>
<tr>
<td>( p,p' )-DDE</td>
<td>( y = 28628x + 4759 )</td>
<td>0.9999</td>
<td>1.56 ppm</td>
<td>7.8 ppb</td>
</tr>
<tr>
<td>( p,p' )-DDT</td>
<td>( y = 1521x + 16.97 )</td>
<td>0.9965</td>
<td>78 ppb</td>
<td>78 ppb</td>
</tr>
<tr>
<td>( p,p' )-DDE</td>
<td>( y = 31396x + 51.98 )</td>
<td>0.9879</td>
<td>7.8 ppb</td>
<td>7.8 ppb</td>
</tr>
</tbody>
</table>

2.3.3.2 GC-MS standard curves for DDT and DDE quantification

In a similar fashion to the linear calibration curves obtained with HPLC-UV, the peak areas over the concentration range of 28 ppb to 45 ppm of \( p,p' \)-DDT and \( p,p' \)-DDE were obtained using GC-MS method 1. Repeatability within one set of triplicate samples was poor at 100 ppm (results not shown), specifically for \( p,p' \)-DDE, most probably due to overloading of the column. Calibration curves were then set from 28 ppb to 45 ppm and gave reasonably good fits at \( R^2 \) =0.999 for \( p,p' \)-DDT and 0.971 for \( p,p' \)-DDE (Figure 2.7).

![Figure 2.7](image)

**Figure 2.7** Standard curves for \( p,p' \)-DDT and \( p,p' \)-DDE obtained with GC-MS method 1. Graph (A) shows the linear regression line though the triplicate repeat of the DDT and DDE calibration curves. (B) The graph with axis converted to log\(_{10}\) scale to show the error, as depicted by 95% confidence interval (vertical lines) at the lower concentrations. Standard solutions (0.156-25 ppm) of each of the compounds were prepared in GC grade dichloromethane and analysed using conditions described in Section 2.2.8.
However, the error in terms of the 95% confidence interval and residuals limited the LOQ to 5.7 ppm for \( p,p' \)-DDT and 2.8 ppm for \( p,p' \)-DDE (Figure 2.7). The lowest concentration at which each of the compounds could be detected (LOD) was approximately 1.4 ppm for \( p,p' \)-DDT and 28 ppb for \( p,p' \)-DDE. The repeatability of specifically the DDT standard curves was questionable and we also found about 1 to 15 ppm DDE (depending on the [DDT] in standard) in the DDT calibration analysis runs (results not shown).

The LOQ and LOD of DDE and DDT, as well as the repeatability on different days were deemed insufficient for our studies. ‘Unknown’ concentration determinations using the standard curves by GC-MS method 1 could not be reliably determined. Only 0.2 µL of the original 1.0 µL injection was analysed due to “split mode” injection. We were therefore unable to determine background levels of the compounds in the unspiked samples. We, however, were confident that the sensitivity could be improved at least 10 times with a full injection, although this analysis mode could entail an extra purification step of the blood and culture samples.

Results from previous studies where standard curves for \( p,p' \)-DDE and \( p,p' \)-DDT were constructed with splitless injection and in some cases with an internal standard, good best fit lines with \( R^2 \) values as high as 0.996-1.000 (Liu and Pleil, 2002; Al-Saleh et al., 2002; Guardino et al., 1996) were obtained. Detection ranges using GC-ECD for separation and GC-MS for identification of these compounds were 0.3 pg instrument detection limit (IDL) and 0.06 µg/L (or 60 ppt) whole blood method detection limit (MDL) for \( p,p' \)-DDE, and 0.7 pg IDL and 0.04 µg/L (or 40 ppt) whole blood for \( p,p' \)-DDT (Guardino et al., 1996).

To optimise the GC-MS analysis further, the concentration range of both \( p,p' \)-DDT and \( p,p' \)-DDE was lowered to between 78 ppt and 10 ppm (Figure 2.8 and Table 2.5), and aldrin was included as internal standard (GC-MS method 2). The standard solutions were prepared from three different stock solutions each of \( p,p' \)-DDE and \( p,p' \)-DDT. The LOD for \( p,p' \)-DDT was found to be 7.8 ppb. However, in light of the fact that the GC-MS method leads to DDT breakdown it was decided to focus this calibration and quantification study on \( p,p' \)-DDE.
Figure 2.8 Standard curve for \( p,p' \)-DDE obtained with GC-MS method 2. A shows the linear regression line through the triplicate repeat of the DDE calibration curve. In B, the graph axes are converted to \( \log_{10} \) scale to show the errors at low concentrations, as depicted by 95% confidence interval (vertical lines). The linear regression line in graphs were fitted with (B) and without (A, B) weighing with \( 1/Y^2 \) to minimise relative square distances. In graphs C and D the prediction of accuracy using the regression line equations (B) is depicted for the \( p,p' \)-DDE calibration curves. All standard curves were analysed as described in Section 2.2.9. Mean ± SEM for three determinations is shown for each data point in B.

Correlation coefficients \( (R^2) \) for the \( p,p' \)-DDE linear calibration curves were \( \geq 0.996 \) thus giving repeatable best fit lines within 95% confidence interval with the use of an internal standard. The residuals for these fits over the whole range, using unweighted regression analysis, showed between 1.0% and 11% residual error for up to 78 ppb. The biggest error in terms of the 95% confidence interval was observed below 78 ppb for \( p,p' \)-DDE (Figure 2.8) if a linear fit over the whole concentration range was considered. Using the unweighted and \( 1/Y^2 \) weighted regression
analysis, the LOQ and LOD of \( p,p' \)-DDE for both analyses were the same at 78 ppb and 78 ppt, respectively, as shown in Figure 2.6 and Table 2.5. This result was comparable to those of Guardino et al. (1996) and the LOD and LOQ drastically improved (refer to Table 2.7 under conclusions).

Analysis results show that the GC-MS method 2 is a significantly improved detection method for \( p,p' \)-DDT and \( p,p' \)-DDE and quantification method for low levels of \( p,p' \)-DDE compared to the GC-MS method 1. Repeatability was also improved with aldrin as internal standard, and analyses data were found to be comparable to that obtained in the HPLC-UV analyses.

*Table 2.5* Summary of data calculated for fitting \( p,p' \)-DDE standard curves from GC-MS method 2 analysis of standard concentration ranges (described in Section 2.3.3.2) using aldrin as an internal standard.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Linear regression equations from fits to standard conc. ranges</th>
<th>( R^2 )</th>
<th>LOQ</th>
<th>LOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>( p,p' )-DDE</td>
<td>Unweighted ( y = 13.69x - 0.04287 )</td>
<td>0.9955</td>
<td>78 ppb</td>
<td>78 ppt</td>
</tr>
<tr>
<td></td>
<td>Weighted with ( 1/Y^2 ) ( y = 12.99x - 0.2040 )</td>
<td>0.9991</td>
<td>78 ppb</td>
<td>78 ppt</td>
</tr>
</tbody>
</table>

### 2.3.4 Quantitative \( p,p' \)-DDT and \( p,p' \)-DDE determination in blood and culture media

The quantitative assessment of \( p,p' \)-DDT and \( p,p' \)-DDE in human blood (packed erythrocytes or whole blood) and RPMI media was done with the three analytical methods. Since DDT breaks down into DDE and DDD, and DDE does not further break down but bioaccumulates in the body’s lipid layers, it was expected that notable levels of DDT and/or DDE would be detected depending on how recently the individuals were exposed or to what levels of DDT they were exposed. As most of the blood was obtained from the Western Cape Blood bank it was expected to detect the compounds since most of the blood samples were donated by South Africans. South Africa used DDT as a pesticide in agriculture and/or for IRS to control malaria during at least the past three decades (Sadasivaiah et al., 2007). Our new HPLC method indicated the presence of DDT and/or DDE levels in certain blood samples as well as RPMI media containing Albumax™. Positive identification and quantitation was done using the GC-MS method 2. The results are given in *Table 2.7*. 

2-29
Background DDT and DDE could not be detected in any of the biological media that was reconstituted to the original extraction solvent volume (4.5 or 6.0 mL) using the GC-MS method 2 analysis (results not shown). Concentrating the dried extracts of the biological samples by reconstituting them in 900 µL solvent to give 5 times concentrated samples enabled us to detect the compounds with the optimised GC-MS method 2. Therefore the increased injection volume to 2 µL with the new GC-MS method 2 compared to 0.2 µL with the GC-MS method 1 improved detection of our compounds in the concentrated samples. The sensitivity of the GC-MS method for DDE allowed for the lower detection limits compared to the HPLC-UV method. The use of concentrated samples and the higher volume injection technique employed for samples analysed by the HPLC-UV and GC-MS (splitless) method 2 allowed the detection of DDT and DDE (Table 2.7).

With the optimised GC-MS method 2 we were able to detect lower levels of DDE in biological samples than the HPLC-UV method (Table 2.7).

Table 2.6 Quantitative DDT and DDE determination in blood and culture media using the HPLC-UV and GC-MS methods as described in Sections 2.2.7 and 2.2.9 respectively.

<table>
<thead>
<tr>
<th>Sample</th>
<th>p,p'-DDT Mean conc. (ppm) ± SD (n)</th>
<th>p,p'-DDE Mean conc. (ppb) ± SD (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HPLC-UV</td>
<td>GC-MS method 2</td>
</tr>
<tr>
<td>Blood donor 1</td>
<td>1.1 ± 0.6 (9)</td>
<td>Trace (6)</td>
</tr>
<tr>
<td>Blood donor 2</td>
<td>not detected (4)</td>
<td>Trace (7)</td>
</tr>
<tr>
<td>RPMI media</td>
<td>2.5 ± 0.8 (7)</td>
<td>Trace (7)</td>
</tr>
<tr>
<td>2 % haematocrit</td>
<td>2.1 ± 1.1 (7)</td>
<td>not detected (5)</td>
</tr>
</tbody>
</table>

The DDE level observed in the blood of donor 1 and one of the batches of donated blood received from the blood bank (254 ± 39 ppb (3)) correlated with the study of Bouwman et al. (1991) showing that the mean blood serum concentrations of DDE in the DDT sprayed areas is 103 ± 85 ppb. Trace or previously undetectable levels of DDT were also recorded with the optimised GC-MS method 2. The higher concentrations recorded for DDT using the HPLC-UV could have been false readings as a result of biological interference. As was previously discussed, the GC-MS method caused degradation of DDT possibly due to the high temperatures
used for analysis. The already low levels of DDT in the biological media were therefore possibly degraded during analysis leaving DDT levels lower than the limit of detection of 7.8 ppb which could explain why we were unable to quantify the compound in the biological media. Results of the determined levels using the two methods for detection are summarised in Table 2.6.

2.4 Conclusions

This study proposes two optimised analytical methods using either HPLC-UV or GC-MS for the determination of different concentration levels of DDT and DDE in blood and malaria culturing RPMI media. Using a cheap, easy and fast liquid-liquid extraction protocol with hexane-ethyl acetate (4:1, v/v) solvent, DDT and DDE were successfully detected and quantified in the biological media. Residual levels of the compounds as a result of previous exposure to DDT were detected with the HPLC-UV and optimized GC-MS method 2. GC-MS, although more costly, was chosen mainly to confirm the identity of the compounds and to detect low levels of the compounds that could not be identified using the HPLC-UV method. A comparative summary of quantitation and detection limits of the three methods used in this study is given in Table 2.7.

Table 2.7 Comparison of the three analytical methods used for determination of \( p,p' \)-DDT and \( p,p' \)-DDE in blood and malaria culturing media as described in Sections 2.2.7 and 2.2.8 and 2.2.9 respectively.

<table>
<thead>
<tr>
<th>Analysis method</th>
<th>( p,p' )-DDT</th>
<th>( p,p' )-DDE</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC LOD</td>
<td>78 ppb</td>
<td>7.8 ppb</td>
<td>Cheap and fast method suitable for analysis of DDT and/or DDE in blood and malaria culturing media in this study.</td>
</tr>
<tr>
<td>HPLC LOQ</td>
<td>1.56 ppm*</td>
<td>1.56 ppm*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>78 ppb**</td>
<td>7.8 ppb**</td>
<td></td>
</tr>
<tr>
<td>GC-MS method 1 LOD</td>
<td>1.4 ppm</td>
<td>28 ppb</td>
<td>Method for analysis of amounts above 5 ppm DDT and DDE in complex biological samples, and identification of unknown compounds.</td>
</tr>
<tr>
<td>GC-MS method 1 LOQ</td>
<td>5.7 ppm</td>
<td>2.8 ppm</td>
<td></td>
</tr>
<tr>
<td>GC-MS method 2 LOD</td>
<td>7.8 ppb</td>
<td>78 ppt</td>
<td>Suitable for the detection of low levels of DDT and DDE, quantification of low DDE levels and identification of unknown compounds.</td>
</tr>
<tr>
<td>GC-MS method 2 LOQ</td>
<td>nd</td>
<td>78 ppb</td>
<td></td>
</tr>
</tbody>
</table>

* unweighted regression analyses; **weighted regression analyses; nd - not determined
The next chapters (Chapter 3 and 4) of this study involve investigating the biological implications of DDT and/or DDE on Plasmodium falciparum chloroquine resistant and sensitive strains. It was therefore imperative for us to develop optimised methods of analysis to determine the DDT and/or DDE contaminant variables in the different compartments of the malaria culture made up of the packed red blood cells, RPMI media and the parasite. The HPLC method and optimised GC-MS method 2 will be revisited and used to determine low levels of DDT and DDE in the three different compartments (media, erythrocyte and parasite) in Chapter 4, while the direct influence of DDT and DDE on parasite viability will be ased in Chapter 3.

2.5 References


Gonçalves, C. and Alpendurada, M. (2002). Multiresidue method for the simultaneous determination of four groups of pesticides in ground and drinking waters, using solid-phase


CHAPTER 3

Anti-malarial activity of \( p,p'\)-DDT and \( p,p'\)-DDE towards chloroquine sensitive and chloroquine resistant strains of *Plasmodium falciparum*

3.1 Introduction

Resistance to conventional antimalarial drugs and insecticides that has emerged over the years has made it the more important to find alternative drugs with different targets, particularly for malaria endemic areas of the world (Ridley, 2002). Insecticides are major contaminants of the environment, with animals and humans being at a high risk of exposure (Noedl *et al.*, 2003). It is therefore imperative to investigate the influence of or role played by chemical compounds, particularly current or previously used insecticides, on the resistance or sensitivity of the already existing antimalarial drugs towards *Plasmodium falciparum* (*P. falciparum*) for drug development purposes.

There are currently several methods that have been developed and are used to assess the effects of drugs and chemicals on the viability of *P. falciparum* parasites (Izumiyama *et al.*, 2009). Traditional *in vivo* methods that measure parasite clearance as sensitivity or three degrees of RI, RII and RIII resistance, have since been replaced by new *in vitro* methods further described below (WHO, 1973; Makler *et al.* 1993; Druilhe *et al.*, 2001; Noedl *et al.*, 2002). Qualitative and quantitative assessments of red blood cell smears with a microscope through parasite counts have also been used but proved to be time consuming, tiring and subjective (Izumiyama *et al.*, 2009).

In 1978, Rieckman developed the original WHO (World Health Organisation) *in vitro* micro-technique defining the minimum inhibitory concentration (MIC) of malaria parasites from clinical isolates (Rieckman *et al.*, 1978; Bacon *et al.*, 2007). This method was developed to perform *in vitro* assays in field conditions, with analysis mainly involving labour-intensive examination of post-culture thick blood smears (Rieckman *et al.*, 1978). Desjardins and colleagues in 1979 then modified the method by measuring the incorporation of radioactive \(^3\)H-hypoxanthine in the growing parasites, and shifting the emphasis of the final estimate of drug susceptibility to 50 % growth inhibition concentration (IC\(_{50}\)) defined as the \( x \)-value (log of drug concentration) of the halfway response between the top and bottom plateaus of the dose response
curves (Desjardins et al., 1979; Du Toit and Rautenbach, 2000). This radioactive-based method has now become the standard measure of in vitro drug susceptibility of antimalarial compounds (Bacon et al., 2007). Colorimetric based incorporation of thymidine analogue, bromodeoxyuridine, in parasite deoxyribonucleic acid (DNA) has also been used as described by Doi et al. (1988). By pulse-labelling the cells with bromodeoxyuridine, those cells synthesising DNA then incorporate bromodeoxyuridine into their DNA (Doi et al., 1988). Elabbadi et al. (1992) developed a method in which radioactive ethanolamine incorporation in phospholipids is measured for the in vitro assessment of antimalarial activity by the general microdilution technique. A large variety of drugs can be tested using radioactive assays since longer and variable incubation times are allowed. Although these assays are faster than the traditional parasite counts due to the high degree of automation and effective in accurately monitoring the in vitro effects of a wide range of antimalarial drugs, even on field isolates, they require relatively high parasite densities of 0.5% (Noedl et al., 2003). They may also be difficult to use in the malaria endemic areas which are mostly resource poor, since these assays have high running costs (Basco, 2007). Specialised disposal systems, well trained personnel, heavy and expensive equipment which is difficult to maintain in tropical areas are required when using the dangerous radioactive material (Druihle et al., 2001; Desjardins et al., 1979; Doi et al., 1988). Several other precursors such as palmitate, serine, choline, inositol and isoleucine have also been used in the radioactive assays (Noedl et al., 2003). As the use of radioactivity in drug assessment assay has inherent safety and cost implications, alternative methods and subsequent modifications were then developed to continuously improve on methods for quantifying parasite growth labour-, time-, cost-, and quality-wise. Colorimetric detection of parasite lactate dehydrogenase (pLDH) activity (Makler and Hinrichs, 1993; Noedl et al., 2003; Ashahi et al., 2005; Druihle et al., 2001) is a widely used method in antimalarial drug discovery research. The Malstat assay which requires an initial parasitemia of between 1-2%, measures pLDH enzymatic activity through colorimetric detection of reduced APAD (3-acetyl pyridine adenine dinucleotide), which in turn reduces blue tetrazolium (nitro blue tetrazolium chloride; refer to Section 3.2.7.1) to form a blue formazan product that can then be measured by spectrophotometry (Basco et al., 1995) as summarised in Figure 3.1. APAD is an analogue of host NAD (nicotinamide adenine dinucleotide), the basis from which the Malstat assay was developed, thus making it a highly
specific assay (Makler et al., 1993). Although the widely used Malstat assay gives reliable and reproducible IC$_{50}$ determinations of compounds in *P. falciparum* parasites, it has been found to be too insensitive for field application (Noedl et al., 2003). A more sensitive assay which measures pLDH levels using specific monoclonal antibodies in what is referred to as the double-site enzyme-linked LDH immunodetection (DELI) assay was therefore developed (Druihle et al., 2001; Piper et al., 1999). The DELI assay can be used in field applications and the assay results are comparable with those obtained for radioactive assays (Noedl et al., 2003). Limited supplies of the monoclonal antibodies have, however, limited the further validation and application of the assay (Noedl et al., 2003).

![Diagram of the Malstat reaction](image)

**Figure 3.1** A diagram showing the Malstat reaction for detecting parasite lactate dehydrogenase. The chemical structure for the tetrazolium dye is shown on the right.

An enzyme linked immunosorbent assay (ELISA) methodology involved in the detection of parasite specific enzyme, histidine rich protein II (HRP2), was developed from an initial HRP2 assay (Noedl H et al., 2002, and Noedl H et al., 2005). Parasite growth and development of the HRP2 assay is measured by the production of HRP2 in a simple, commercially available double-site ELISA test kit (Noedl H et al., 2003). The HRP2 assay is roughly 10 times more sensitive than the radioactive assays, and it requires less complicated equipment. Slow acting drugs can also be tested because of the long incubation periods of 48 to 72 hours (Noedl H et al., 2003). Since the HRP2 assay was relatively expensive, a cheaper and more generic antigen capture HRP2 ELISA assay was developed. Two commercial monoclonal antibodies are used for this
drug sensitivity test with cheaper alternative test kits compared to the one enzyme specific antigen used in the initial HRP2 assay (Noedl H et al., 2005). DNA staining with fluorescent based SYBR Green 1 (Figure 3.2), Pico green or YOYO-1 dyes have been successfully used in several studies. Fluorescent nucleic acid intercalating dyes are used in the measurement of in vitro malaria growth inhibition. Since mature erythrocytes do not have RNA and DNA, the dyes bind specifically to malaria DNA in any erythrocytic stage of parasite development. The dyes, however, preferably bind to the double stranded DNA, compared to the single stranded RNA. (Bennett TN et al., 2004; Smilkstein M et al., 2004; Bacon DJ et al., 2007; Johnson JD et al., 2007, and Rason MA et al., 2008; Quashie et al., 2006; Corbette et al., 2004). Although initial experiments with the fluorescence-based assays required complex, multistep protocols, they are accurate, reliable and cheaper than the radioactive and antibody-based assays, as well as easy to perform, mostly requiring one step in the plate before obtaining the results. These assays have also been improving of late and are used in high-throughput screening (Co et al., 2010). The SYBR Green 1 assay makes in vitro drug sensitivity testing more affordable for researchers in malaria endemic countries, as this assay is significantly cheaper than most other assays discussed above (Bacon et al., 2007; Kaddouri et al., 2006; Smilkstein et al., 2004). The study by Rason and others (2008) reiterates the reliability of the SYBR Green 1 method for easy, fast, cheap and simple to use in vitro assessments of P. falciparum parasites (Rason et al., 2008).

Figure 3.2 Structure of parasite DNA binding SYBR Green 1 dye used in the fluorometric detection of parasite DNA in the SYBR Green 1 assay as further described in Section 3.2.7.2.
Although flow cytometry (FCM) analysis using the different fluorescence DNA intercalating
dyes has not become a popular method of analysis used, it has proved to be useful in analysing
blood stage parasites and to be highly comparable with other methods such as microscopy,
hypoxathine uptake and pLDH assays (Janse and Vianen, 1994; Jouin et al., 1995; Persson et al.,
2008; Persson et al., 2006; Bergmann-Leitner et al., 2006; Sanchez et al., 2007; Contreras et al.,
2004; Shi et al., 1999). Malaria is detected in each cell by using flow cytometry which yields
information on forward scatter, which is relative to size, and side scatter, which is relative to
granularity, and fluorescence, with minimal time and effort. The dyes used in FCM are cheap.
The method has been shown to be sensitive and specific to as low as 0.002-0.003% parasitemia,
and as a reliable means to conduct drug sensitivity assays (Co et al., 2010). The equipment is,
however, expensive and non portable although low cost, compact and simpler flow cytometers
that are practical for field use are becoming available as a result of recent innovations (Co et al.,
2010). Most recently, Ku et al., (2011) introduced quantum dots as a new tool for use in
antimalarial drug assays, and from there developed a rapid and efficient assay to screen
antimalarial compounds. PEGylated-cationic quantum dots, which are fluorescent nanocrystals,
are used as probes to directly label *P. falciparum* infected erythrocytes with late stage parasites
in an immunofluorescence antimalarial drug-screening assay. Apart from being suitable for high-
throughput screening, this method only requires an incubation period of 24 hours and shows 50%
Improved sensitivity compared to the pLDH assay in detecting drug efficacy within a malaria
parasite cycle (Ku et al., 2011).

It may at times be extremely difficult to compare drug IC\textsubscript{50} values directly from laboratory to
laboratory due to the many variations in protocol details (Basco, 2004). To overcome this
challenge, standardised culture systems and quality controls are recommended (Basco, 2003).
Choosing a particular assay appropriate for particular laboratory settings and maintaining
consistency in the assay method goes a long way in giving reproducible results (Bacon et al.,
2007). We therefore decided to make use of two complementary assays in this study, the parasite
lactate dehydrogenase (Malstat) assay and the SYBR Green 1 assay, to determine the *in vitro*
antimalarial activities of dichloro-diphenyl-trichloroethane (\(p,p'\)-DDT) and dichloro-diphenyl-
dichloroethylene (\(p,p'\)-DDE) towards chloroquine sensitive (CQS) *P. falciparum* D10 and
chloroquine resistant (CQR) *P. falciparum* Dd2 strains respectively.
Although CQ resistance only evolved after exposure to the drug, the resultant multiple resistance profiles of some of the mutated transporters, as described in Chapter 1, indicated that they can accommodate a wide spectrum of organic molecules, many of which contain a phenyl-chloride moiety. DDT and its major metabolite DDE contain two such moieties (Figure 3.3) and could possibly be accommodated by these transporters.

Figure 3.3 Chemical structures of DDT, DDE and CQ.

In this study, the activity of \( p,p' \)-DDT and \( p,p' \)-DDE on CQS and CQR \( P. falciparum \) is determined to assess if resistance towards CQ is a factor in their activity or inactivity. There is no published study, to our knowledge, on the effect of DDT and DDE on erythrocytic stages of \( P. falciparum \) or any other malaria strains as the pesticide has only been known to act against the mosquito vector and not the malaria parasite. As is discussed in Chapters 2 and 4 it is highly likely that there are persistent low levels of DDT and/or DDE present in the malaria vector and the human host due to the long half lives and weak biodegradable nature of these compounds. Therefore, the question of whether the presence of such aromatic organochlorides places selective pressure on malaria strains, possibly the more resistant strains that can cope with DDT and/or DDE arises. Here we report the first part of a two part study to address the research question.

### 3.2 Methods and materials

#### 3.2.1 Antimalarial drugs used in this study

Chloroquine diphosphate salt (CQ, Mr 515.87), \( p,p' \)-DDT (Mr 354.49), \( p,p' \)-DDE (Mr 318.03) and all reagents used in this study were purchased from Sigma-Aldrich (Steinheim, Germany) for use in the \textit{in vitro} tests unless otherwise stated. All three compounds/drugs were analytically...
weighed as described in Section 2.2.3 of Chapter 2 and stored in a desiccator at room temperature until required for use.

Stock solutions of the analytically weighed \( p,p'\)-DDT and \( p,p'\)-DDE were prepared a day before conducting the assays in a minimum amount of filtered HPLC grade methanol to achieve a final concentration of 5.00 mg/mL for each of the compounds.

The stock solutions were diluted to 1.00 mg/mL in complete RPMI media. Eight doubling dilutions of \( p,p'\)-DDT and \( p,p'\)-DDE in complete RPMI medium (1.00 mg/mL to 7.80 µg/mL) were prepared in glass vials cleaned, as described in Section 2.2.3 of Chapter 2, a day before conducting the dose-response assays and stored at 4°C until required. The solutions were prepared in RPMI media so as to reduce the amount of methanol to less than 2% in the malaria cultures to prevent exposure of the parasites to high methanol concentrations.

CQ stock solutions were prepared in analytical quality water (deionised water filtered through a MilliQ\textsuperscript{TM} system from Millipore-Waters) to starting concentrations of 5.00 µg/mL. Aliquots of 1.00 µg/mL or 5.00 µg/mL of the CQ stock solution were stored in Eppendorf tubes at 4°C. Doubling dilution solutions (1.00 µg/mL to 7.80 ng/mL) were then prepared from the 1.00 µg/mL stock solution in analytical quality water in dilution plates on the day of the dose response assay for D10 parasites. A fivefold higher concentration range was used for the CQR strains (Dd2 and D10r).

### 3.2.2 Blood samples in malaria parasite culturing

For culturing, we used packs of 300 mL donated whole blood stored in 63.0 mL citrate phosphate dextrose anticoagulant from the Western Cape Blood services (or National Health Laboratory Services in South Africa), containing enriched erythrocyte fraction in 100 mL saline-adenine-glucose-mannitol red blood cell preservation solution. This additive solution also lowers the red blood cell (RBC) unit haematocrit (0.6±0.1 L), and stores for up to 42 days. Fresh erythrocyte enriched blood, however, was obtained fortnightly as it was observed that \( P. falciparum \) cultures do not grow as well on older erythrocytes. The erythrocytic fraction from A\textsuperscript{+} blood group was routinely used for the \( P. falciparum \) parasite culturing, except when stated otherwise. For culturing purposes, all blood samples were washed twice in RPMI culture media.
by centrifuging at $1300 \times g$ for 5 minutes per wash, and removing the plasma and buffy coats, if present.

Blood used for all sets of experiments in this study was completely anonymous left over human blood complying with relevant legislation. There was therefore no breach of ethics or approval required. Alternatively the researcher involved in this study used her own blood, drawn by qualified medical personnel, for analysis and culturing purposes.

3.2.3 Parasite culturing and enrichment

Culture derived asexual erythrocytic stage *P. falciparum* parasites, namely, CQS *P. falciparum* D10 and CQR *P. falciparum* Dd2, were kindly provided by Prof. Peter Smith from the University of Cape Town, Division of Pharmacology.

The cultures were initiated from glycerol stocks preserved first in liquid nitrogen then stored at -80°C as described in Section 3.2.6. Using the methods of Trager and Jensen (1976), and Lambros and Vanderberg (1979), the parasites were maintained in a synchronised continuous culture.

After the parasites were thawed, they were resuspended in 10.4 g RPMI 1640 medium supplemented with 4 g glucose, 6 g HEPES, 5 g albumax II, 0.4 g hypoxanthine (first dissolved in 1 mL sodium hydroxide before adding to the media), 50 mg gentamicin and 2.1 g sodium bicarbonate all made up in 1 L analytical quality water (Cranmer *et al.*, 1997; Trager and Jensen, 1976). The media was set to a pH of 7.2-7.3, filter sterilized using a 0.22 µm filter and stored at 4°C in a sterile bottle, previously autoclaved twice. All the chemicals used to prepare the media were purchased from Sigma-Aldrich (Steinheim, Germany).

The cultures were maintained in sterile red standard cap 250 mL Cellstar tissue culture flasks (Greiner Bio-One GmbH, Germany) in a total volume of 50 mL medium, and incubated at 37°C under a gas mixture of 3% O₂, 4% CO₂, and 93% N₂, without shaking.

A 3-4% haematocrit was maintained for all cultures grown under the above mentioned conditions. Fresh uninfected erythrocytes were added every two days and the RPMI media refreshed daily until approximately 5-15% parasitemia was reached. The parasites were grown
continuously, but not for more than 3 months from one starter culture to prevent genetic alteration. Cultures with high parasitemia (>10%) were either frozen away in glycerol stocks at -80°C to maintain culture collection or used for dose response assays.

3.2.4 In vitro synchronisation of ring stage parasites
For the in vitro synchronisation (Lambros and Vanderberg, 1979) of ring stage parasites, 10.0 mL of filter sterilised 5% D-sorbitol solution was added to pelleted erythrocytes under sterile conditions, and the mixture was carefully mixed by inversion of the falcon tube. The mixture was incubated at 37°C for 5 minutes, sedimented at 750×g for 3 minutes, and the erythrocytes were resuspended in RPMI medium and returned to the flask before being flushed with the gas mixture as described in Section 3.2.3.

3.2.5 Parasite counts using Giemsa staining
To assess the asexual stage the parasites, a drop of sedimented erythrocytes from the culture was picked up using an autoclaved Pasteur pipette and placed onto a microscope slide. A smear was prepared using a second slide. The smear was allowed to dry before being fixed onto the slide by immersing the slide briefly in ethanol and again allowing it to dry.

DNA interchelator Giemsa stain mixture from Sigma-Aldrich (Giemsa stain diluted approximately 1:10 in phosphate-buffered saline, pH 7.2) was used to cover the fixed blood smear for 2-5 minutes to stain the parasite DNA. The stain was washed off with water and air dried before viewing it under oil immersion at the 100× objective lens under the microscope (Reilly et al., 1997).

Parasitemia was calculated by counting the number of parasites in the infected erythrocytes in relation to the normal erythrocytes and it was expressed as a percentage. The percentage parasitemia of at least three different positions on the slide was calculated, and the average of the three percentages taken as the final % parasitemia.

3.2.6 Freezing and thawing of malaria parasites
Only ring stage parasites were frozen as their erythrocyte host membranes are more robust. The parasites were frozen away at a high parasitemia greater than or equal to 10% according to the method by Diggs et al. (1975).
Glycerol medium in which the parasites were frozen was prepared by first dissolving 1.6 g sodium lactate, 30 mg potassium chloride, 1.38 g sodium hydrogen phosphate and 57 g glycerol (AnalaR grade, BDH Chemicals Ltd) in about 30 mL analytical quality water, setting the pH to 6.8, then making up the solution to 100 mL with analytical quality water before filter sterilising the medium with a 0.22 µm filter.

The volume ratio of packed erythrocytes to glycerolyte medium required is 3:5. One volume glycerolyte medium was added drop wise onto the pellet using a sterile syringe while constantly swirling the tube under sterile conditions. The mixture was allowed to stand for 5 minutes, and the remaining glycerolyte medium was then added slowly while swirling. The mixture was aliquoted (2 mL each) into sterile Cryo.s PP tubes (Greiner Bio-One GmbH, Germany) and stored at -80°C. The tubes containing the parasites in glycerol stock solutions were first transferred to liquid nitrogen briefly before being stored in -80 °C until they were required for use.

To thaw the parasites, three solutions were each made up in analytical quality water. Solution A consisting of 12 % NaCl, solution B containing 1.8 % NaCl, and solution C with 0.9 % NaCl plus 0.2 % glucose (Diggs et al., 1975). All three solutions were filter sterilized using a 0.22 µm filter and stored at 4-8 °C until required for use. The cells were first thawed in the Cryo.s PP tubes at 37 °C in a water bath before being transferred to a sterile 50 mL centrifuge tube under sterile conditions in a laminar flow cabinet. To gradually reduce the osmotic potential of the thawed freezer stock, the parasites in glycerol stock solutions were diluted in solution A to a volume ratio of stock to solution A of 5:1. Solution A was added drop wise using a sterile syringe while swirling and was allowed to stand for 5 minutes before slowly adding 10 mL of solution B. The mixture was then centrifuged for 5 minutes at 400×g. The supernatant was aspirated using a vacuum source and 10 mL of solution C was slowly added to the pellet with gentle swirling. Again, the mixture was centrifuged for 5 minutes at 400×g, the supernatant aspirated, and the pellet washed with 20 mL media before spinning for another 5 minutes at 400×g (Diggs et al., 1975).
The parasite containing pellet was reintroduced into culture with complete media and gassing as described in the culturing procedure in Section 3.2.3. At this stage, the culture was left for 3-4 days at 37°C without changing the medium to allow the parasites in culture to recover.

### 3.2.7 Parasite viability and dose response assays

After being thawed, the cultured parasites were generally grown for about 10 to 14 days until the parasitemia reached roughly 5 to 15% and the parasites had adapted well enough to be used for the drug sensitivity assays.

Once the well synchronized parasites in young trophozoite stage reached 5-10%, the infected erythrocytes were centrifuged at 750×g for 3 minutes. The supernatant was aspirated and an aliquot of the infected erythrocytes was diluted in fresh uninfected erythrocytes before being suspended in RPMI medium to achieve a final 2% parasitemia and 2% haematocrit suspension. The level of parasitemia of an aliquot of a stock culture was measured by light microscopy following Giemsa staining as described above in Section 3.2.5.

Dose response assays were then carried out as described by Desjardins and colleagues (1979). Using sterile untreated 96-well flat bottom polystyrene microtiter plates from NUNC (Denmark) 90 µL of the 2% parasitemia and 2% haematocrit suspension in early trophozoite stage was added to each of the wells (Rautenbach et al., 2007; Wiehart et al., 2006).

An aliquot of 10 µL per well of the drugs prepared from the serial dilutions as described in Section 3.2.1 were added in triplicate to achieve a final volume of 100 µL in each well (Rautenbach et al., 2007; Wiehart et al., 2006). The drugs were therefore diluted tenfold in the total 100 µL of culture to achieve the desired final concentrations for the drugs in the CQS and CQR strains.

Assay blanks or background were calculated from wells that received erythrocytes with 2% parasitemia plus 10 µL of 200 µg/mL gramicidin S (GS, Mr 1140.6) (Rautenbach et al., 2007). Infected erythrocytes without the drug added were used as the growth controls for the assay. Growth in the presence of drug solvents was determined from wells that received the 2% haematocrit, 2% parasitemia suspension with 10 µL of analytical quality water or 2% methanol, neither of which caused parasite growth inhibition (results not shown).
The plates were placed in a sealed desiccator used as the incubation chamber that had been thoroughly sterilised with isopropanol and left overnight under direct UV light before being used the following day. After the plates were placed in the incubation chamber, it was flushed for 5-10 min with the gas mixture consisting of 3% O₂, 4% CO₂, and 93% N. The chamber was then sealed and the plates were incubated at 37°C for 48 hours.

After the 48 hour incubation period, the plates were directly frozen at -20°C, until development by Malstat (lactate dehydrogenase activity) and SYBR Green 1 ([DNA]) assays. The dose response assays were all carried out at least in triplicate using the same culture on the same day (technical repeats). Similar experiments were repeated on at least three different days with a different culture under the same conditions (biological repeats) to check the reproducibility of results.

### 3.2.7.1 Colorimetric detection of parasite lactate dehydrogenase using the Malstat Assay

After the 48 hour incubation of the plates at 37°C and freezing away at -20°C the plates were thawed at room temperature. The parasite lactate dehydrogenase assay adapted from Gomez et al. (1997) was used.

Malstat solution was prepared from 200 µL Triton X-100 (BDH Laboratory Supplies, Poole, England), 2 g L-lactic acid as substrate, 0.66 g Tris-HCl buffer (from Boehringer Mannheim, or Roche, ) and 0.011 g of 3-acetylpyridine adenine dinucleotide (APAD) as coenzyme, in 100 mL analytical quality water. The solution was set to a pH of 9.0 (Nkhoma S et al., 2007). A second solution, NBT/PES solution consisting of 1.96 mM nitro blue tetrazolium (NBT) and 0.24 mM phenazine ethosulfate (PES) was used to initiate the lactate dehydrogenase reaction. This solution was stored in an aluminium foil covered falcon tube at 4-8 °C until required.

Aliquots of 100 µL Malstat solutions were pipetted into untreated 96-well flat bottom polystyrene microtiter plates (Greiner Bio-One GmbH, Germany). The thawed haemolysed suspensions in each of the wells in the dose response assay plate were mixed by pipetting the suspensions up and down. An aliquot of 15 µL of the haemolysed suspensions were then added to the 100 µL Malstat solution, followed by 25 µL NBT/PES solution to initiate the lactate dehydrogenase reaction.
After the contents of each well were properly mixed, the plates were then incubated at room temperature in the dark for 30 minutes. The reduced form of APAD formation was measured by colorimetric determination at 650 nm using a Model 680 Microplate reader from Biorad.

Air bubble interferences were avoided by gently blowing on the plate with a hairdryer to get rid of all air bubbles in the wells. All the chemicals used for preparation of reagents were purchased from Sigma-Aldrich (Steinheim, Germany) unless otherwise stated.

3.2.7.2 Fluorescent detection of parasite DNA using the SYBR Green I Assay

Following incubation, the dose response assay plates were frozen and stored at –20°C until the SYBR Green I assay was performed using the procedure described by Bennett and colleagues (2004).

The plates were thawed at room temperature and each sample was mixed by pipetting up and down using a multipipette until the thawed culture was well mixed. Aliquots (90 µL) of the mixed culture were transferred to a black 96-well sterile NUNC flat bottom LumiNunc/FluoroNunc microplate which aids in reducing fluorescence cross talk between wells thereby improving assay reproducibility.

A solution of SYBR Green I, diluted to a 20× concentration in a phenol red free complete medium was prepared. The solution was prepared by adding 100 µL of the original SYBR Green I nucleic acid gel stain, which according to the manufacturer’s instructions was 10 000× concentrated in dimethyl sulphoxide (DMSO), to 50 mL lysis buffer. The lysis buffer consisted of 20 mM TRIS hydrochloride (pH 7.5) and 5 mM EDTA to give the desired 20× concentration of the SYBR Green I solution according to the manufacturer’s instructions. The SYBR Green I solution was stored in an aluminium foil covered falcon tube at 4-8°C until required for use.

The SYBR Green I solution was then added to each well by use of a volume equal to 10 % of the final liquid volume in the well (Bennett et al., 2004) to start the reaction. Therefore, 90 µL of the culture was transferred into a black 96-well plate, followed by the addition of 10 µL of the 20× SYBR Green I solution in lysis buffer. The contents of each well were properly mixed and the plates were covered in aluminium foil and incubated 37°C for 30 minutes in the dark to develop since the SYBR Green I dye is sensitive to light.
The SYBR Green I dye fluorescence emission at 521 nm was measured from the plates at an excitation wavelength of 490 nm using a Varioskan plate reader utilising SkanIt Software 2.4.1. Air bubble interferences in the wells were avoided as described above.

### 3.2.8 Data processing

Total growth was determined from wells that received only the 2% haematocrit, 2% parasitemia culture with 10 µL of analytical quality water or ≤ 2% methanol which was shown to have no measureable effect on parasite viability. Background absorption of fluorescence was calculated from wells that received the infected erythrocytes plus 10 µL of 200 µg/mL GS. The absorption values recorded in each well containing the drug were converted to percentage inhibition as described by Rautenbach et al. (2006) using the following:

\[ \text{Equation 3.1:} \]

\[
\% \text{ growth inhibition} = 100 - \frac{100 \times (\text{Absorption of well} - \text{Average Absorption of background} )}{\text{Average Absorption of growth wells} - \text{Average Absorption of background}}
\]

For the SYBR Green fluorescence assays the absorption values in Equation 3.1 were substituted with the measured SYBR Green 1 dye fluorescence.

Non-linear regression of Graphpad Prism 4.0 (Graphpad Software, San Diego, USA) was used to analyse all dose response data obtained from the Malstat and SYBR Green 1 assays. Sigmoidal dose response curves with variable slope were fitted to each of all data sets as described by Du Toit and Rautenbach (2000) and Rautenbach et al. (2006) using

\[ \text{Equation 3.2:} \]

\[
Y = \frac{\text{bottom} + (\text{top} - \text{bottom})}{1 + 10^{\log IC_{50} \times \text{Activity slope}}}
\]

The mean values for each data set points without weighting were considered for curve fitting. The 50 % growth inhibitory concentration (IC$_{50}$) was then calculated from on the x-value (log of drug concentration) of the halfway response between the top and bottom plateaus of the dose response curves. The experiments were performed at least in triplicate at each concentration for each of the compounds against the target cells.
3.3 Results and discussion

3.3.1 Comparison of Malstat and SYBR Green 1 assays
Both the Malstat and SYBR Green 1 assays gave comparable dose response and inhibition parameter results for GS and CQ towards *P. falciparum* D10 cultures (Figures 3.4A and B). Both assays were highly repeatable and the sigmoidal dose response curves constructed using *Equation 3.2* were with $R^2 > 0.99$ and residual error falling between -5 and 2.5% (Figure 3.4).

**Figure 3.4** Combined dose-response curves obtained with trophozoite infected red blood cells as measured after 48 hours. Percentage growth inhibition was determined using colorimetric based Malstat assay and parasite DNA fluorescence detection using the SYBR Green 1 assay. *P. falciparum* CQS D10 infected cells were treated with serial dilutions of GS (A) and CQ (B). The graph under each dose response graph shows the residuals for the sigmoidal line fits which were used to determine inhibition parameters. The mean of 24-28 determinations for the Malstat assay (6-7 biological repeats) and 15-18 (5-6 biological repeats) for the SYBR Green 1 assay of each data point is shown with error bars indicating the 95% confidence interval (CI).
However, the individual repeats, encompassing technical and biological repeats of the SYBR Green 1 assay compared to the Malstat assay were more prone to error as can be seen by the generally larger 95% CI (Figure 3.4). This is most probably due to the difference between the two assays. The SYBR Green 1 dye binds to DNA from any source and fluorescence is measured. Any contamination DNA from the environment, differences in DNA concentration between parasite stages and compounds that may quench fluorescence or fluoresce themselves will influence the result (Co et al., 2010). Presence of haemoglobin which has a wide absorption spectrum has also been known to possibly interfere with the emission of SYBR Green 1 in the fluorescence based assay (Co et al., 2010). The Malstat assay is highly specific for the parasite lactate dehydrogenase (pLDH), but can also be prone to error in cultures that are desynchronized, as the ring stages have less pLDH activity than the trophozoite and shizont stages (Makler et al., 1993a; Makler et al., 1993b). The difference between the two assays at low CQ concentrations resulting in a lower “bottom” indicating higher growth as determined by the SYBR Green 1 assay compared to that of the Malstat assay may have been due to the nature of the assays as described above.

Neither of the two assays showed that CQ reached a 100% inhibition of parasite growth and in all of the assays with CQ as inhibiting drug an inhibition plateau was reached between 75 and 90% growth inhibition (Figure 3.4). This could be due to the selection and survival of persistent or CQ resistant parasites during the assay period.

Comparison of the IC$_{50}$ inhibition parameter from the individual biological repeats and different assays again showed the SYBR Green assay gave more variable data with both GS and CQ as antimalarial compounds, if the 95% CI are used as the repeatability indicator. (Figure 3.5). With the acquisition of fresh blood for our cultures and assays, blood donors change every 14 days, to which some of the intra assay variability could be attributed.

The SYBR Green 1 assay is strongly recommended by some researchers for in vitro assays in research laboratories of malaria endemic areas (Bacon et al., 2007; Kaddouri et al., 2006; Smilkstein et al., 2004). This assay proved to be a technically easier assay, but more prone to error. From the above arguments on the repeatability, errors and subtle differences we have
observed between the two assays, we decided to employ both assays in our studies to determine the inhibition parameters and parasite survival in the presence of \( p,p' \)-DDT and \( p,p' \)-DDE.

![Graph showing the comparison between the IC\(_{50}\)s determined over a period of at least two years using the two assays and two test compounds.](image)

**Figure 3.5** Bar-graph showing the comparison between the IC\(_{50}\)s determined over a period of at least two years using the two assays and two test compounds. The number above each bar represents the biological repeats and the error bar the 95% CI.

### 3.3.2 Effect of Sorbitol on activity of CQ in PfD10

In order to ensure that the assays are conducted on synchronised cultures the parasite cultures were synchronised with D-sorbitol. Only rings survived the synchronisation procedure since their membranes are more robust. Trophozoites and schizonts were destroyed by the sorbitol induced lysis (Diggs *et al.*, 1975).

During the run of this project an unexpected increase in CQ inhibition parameters was observed for some of our cultures, while the inhibition parameter against GS remained within the expected range. D-Sorbitol synchronised CQS D10 cultures, synchronised every cycle (48 hours) for roughly 2 months, showed reduced CQ activity resulting in a roughly 2 to 3.5 times increase in the CQ IC\(_{50}\). A progressive shift in the CQ dose response curve to the right in relation to an unstressed culture synchronised once a week indicated possible resistance (refer to **Figure 3.8** below). The parasite culture from this sorbitol synchronisation regimen remained stable in terms of CQ resistance and was named *P. falciparum* D10r. Frequent synchronisation could have led to the selection of more CQ resistant parasites which continued to be cultured.
We also found that a 1000 fold lower concentration than used in the synchronisation, namely 0.5 mg/mL sorbitol almost totally antagonised the activity of 0.025 to 1 μg/mL CQ towards the D10 strain (Figure 3.6). There was no significant difference in percentage inhibition between 0.5 mg/mL D-sorbitol alone and in combination with 48 nM (25 ng/mL) CQ. This combination gave approximately the same percentage inhibition at which 12.5 ng/mL CQ alone inhibits growth of the D10 strain, indicating a >50% loss of activity (Figure 3.6). At higher concentrations of 97 and 194 nM (50 and 100 ng/mL) CQ, even more pronounced antagonism was observed in the presence of 0.5 mg/mL D-sorbitol, with a significant activity loss of ±55% and ±65% respectively. The CQ activity could not be regained even at 1.9 μM (1 μg/mL) in the presence of 0.5 mg/mL sorbitol (results not shown). This indicated that the 0.5 mg/mL sorbitol supports parasite growth at high CQ concentrations of up to 1.9 μM (1000 ng/mL) CQ and may assist in the selection of CQ-resistant parasites with 2-3 fold reduced CQ (Figure 3.8).

![Figure 3.6 Comparison of percentage growth inhibition of D10 strain at different concentrations of CQ with or without 0.5 mg/mL D-sorbitol as measured after 48 hours using the Malstat assay. The mean of 9-18 repeats for the sorbitol exposed parasites and 24 repeats for the parasite exposed to CQ alone and standard error with the mean (SEM) is shown for each data point. Statistical analysis was done with Bonferroni post test using a 2-Way ANOVA analysis of the data.](http://scholar.sun.ac.za)

From these results it is clear that care must be taken in the synchronisation regime as sorbitol can influence CQ activity, however, desynchronised cultures will in turn influence repeatability of assays. We therefore opted to use a regime of once a week synchronisation and synchronising four days before assays.
3.3.3 Assay quality control using GS towards *P. falciparum* CQR and CQS strains

In order to assess if culture and assay conditions remained constant for each group of assays, the activity of GS was also determined against all three strains. GS was used as an assay indicator compound due to its selective haemolytic activity towards trophozoite infected erythrocytes (Rautenbach *et al.*, 2007). As all our assays were done with synchronized parasites in the trophozoite stage, it was expected that GS IC$_{50}$ would be similar for all the three *P. falciparum* strains. GS showed high activity against all the strains, with all three strains giving similar IC$_{50}$s ranging from 1.2 -1.5 µM (*Figure 3.7 and Table 3.1*). These values correlate well with the GS IC$_{50}$ values reported in literature (Rautenbach *et al.*, 2007).

*Figure 3.7* *P. falciparum* D10, D10r and Dd2 infected cells treated with serial dilutions of GS. Graphs A and B show the GS activity against the three strains determined using the Malstat (top graph) and SYBR Green 1 (bottom graph) assays, respectively. The average of at least 16 to 28 determinations (4 to 8 biological repeats) of each data point is shown with error bars depicting SEM.
If the GS IC$_{50}$ remained within a narrow error limit for a specific group of dose response assays, this confirmed that assay conditions in terms of erythrocyte concentration and parasitemia were comparable between assays and strains.

**3.3.4 Activity of CQ, p,p’-DDT and p,p’-DDE towards P. falciparum CQR and CQS strains**

Parasite inhibitory concentrations (IC$_{50}$) of CQ were observed to be from 38-40 nM with both the Malstat and SYBR Green 1 assays in *P. falciparum* D10 (*Figure 3.8*).

![Figure 3.8 P. falciparum D10, D10r and Dd2 infected cells treated with serial dilutions of CQ. Graphs A and B show the activity of CQ towards the three strains determined using the Malstat and SYBR Green 1 assays, respectively. The average of at least 6-24 determinations (2-8 biological repeats) of each data point is shown with error bars depicting standard error with the mean (SEM).](http://scholar.sun.ac.za)

The CQ IC$_{50}$ values (*Table 3.1*) were comparable to those reported in literature (Graziose *et al*., 2011, Saliba *et al*., 1998, Fivelman *et al*., 1999). The activity of CQ towards the CQR Dd2 strain
was observed to be 277-298 nM for IC$_{50}$ (Table 3.1) which was also comparable to those values reported in literature (Graziose et al., 2011). The sorbitol influenced D10r parasites had an IC$_{50}$ of 98-132 nM. While the IC$_{50}$s for CQ in the 3 strains were significantly different (P<0.001) with that of Dd2>D10r>D10 (Table 3.1), the IC$_{50}$s were comparable (P>0.05) with the two assays. Based on the mode of action of CQ in the parasite, high accumulation of CQ into the food vacuole of CQS D10 strain allows CQ to effectively kill the parasites (Petersen et al., 2011). Effective expulsion of the CQ from the vacuole or its limited ability to enter the food vacuole to target the parasites probably led to the higher IC$_{50}$s observed for D10r and CQR Dd2 strains. P. falciparum CQ resistance is generally the consequence of a decrease in accumulation of CQ in the parasite vacuole and linked to the expression of two membrane bound transporters, namely PfCRT and PfMDR-1 (Petersen et al., 2011).

Table 3.1 Summary of the in vitro antimalarial activity parameters of CQ, p,p’-DDT, p,p’-DDE and GS towards P. falciparum determined by the Malstat (top value) and SYBR Green 1 (bottom value) assays.

<table>
<thead>
<tr>
<th>Compound</th>
<th>D10</th>
<th>P. falciparum strain</th>
<th>Dd2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC$_{50}$±SEM (n)</td>
<td>IC$_{50}$±SEM (n)</td>
<td>IC$_{50}$±SEM (n)</td>
</tr>
<tr>
<td>GS (µM; µg/mL)</td>
<td>1.37±0.2; 1.56±0.3 (8)</td>
<td>1.49±0.09; 1.70±0.1 (4)</td>
<td>1.26±0.09; 1.44±0.1 (6)</td>
</tr>
<tr>
<td></td>
<td>1.46±0.3; 1.67±0.4 (7)</td>
<td>1.18±0.03; 1.35±0.03 (2)</td>
<td>1.17±0.2; 1.33±0.2 (5)</td>
</tr>
<tr>
<td>CQ (nM; ng/mL)</td>
<td>40.0±3.4; 20.6±1.8 (12)</td>
<td>132±23; 68.1±11.9 (3)*</td>
<td>277±18; 143±9.3 (6)*</td>
</tr>
<tr>
<td></td>
<td>38.9±4.9; 20.1±2.5 (5)</td>
<td>98.0±0.14; 50.6±0.1 (2)*</td>
<td>298±19; 154±9.8 (5)*</td>
</tr>
<tr>
<td>p,p’-DDT (µM; ppm)</td>
<td>22.8±4.4; 8.09±1.6 (9)</td>
<td>19.3±0.08; 6.86±0.03 (3)*#</td>
<td>23.2±3.4; 8.23±1.2 (6)*#</td>
</tr>
<tr>
<td></td>
<td>28.1±3.9; 9.96±1.4 (10)</td>
<td>13.7±1.3; 4.84±0.46 (2)*#</td>
<td>31.9±16.1; 11.3±5.7 (5)*#</td>
</tr>
<tr>
<td>p,p’-DDE (µM; ppm)</td>
<td>36.2±7.9; 11.5±2.5 (7)</td>
<td>20.3±0.66; 6.46±0.21 (3)*#</td>
<td>22.9±3.1; 7.29±0.99 (6)*#</td>
</tr>
<tr>
<td></td>
<td>31.5±5.0; 10.0±1.6 (8)</td>
<td>24.4±1.4; 7.75±0.43 (2)*#</td>
<td>27.4±7.6; 8.7±2.4 (5)*#</td>
</tr>
</tbody>
</table>

n = number of independent experiments/biological repeats performed at least in triplicate technical repeats for each of the compounds; ppm = concentration in parts per million or µg/mL;
*P<0.001 for CQ IC$_{50}$ compared to activity towards D10 strain, #P>0.05 for p,p’-DDT and p,p’-DDE IC$_{50}$ compared to activity towards D10 strain

Using both the Malstat and SYBR Green 1 assays described in Sections 3.2.7.1 and 3.2.7.2 respectively, the in vitro IC$_{50}$ values of p,p’-DDT and p,p’-DDE in the CQS Plasmodium
*falciparum* D10 and CQR Dd2 strains were determined for the first time to our knowledge (Figure 3.9, Table 3.1).

*p,p’*-DDT and *p,p’*-DDE had equally high activity towards all three strains. Their IC\textsubscript{50}s were in the range of 22-28 µM and 31-36 µM, respectively towards D10 parasites, 13-19 µM and 20-24 µM respectively against D10r parasites, and 23-31 µM and 22-27 µM, respectively towards the Dd2 parasites (Table 3.1). Although lower IC\textsubscript{50} and IC\textsubscript{max} values were recorded towards the CQR strains the differences were not significant (P>0.05).

Figure 3.9 *P. falciparum* D10, D10r and Dd2 infected cells treated with serial dilutions of *p,p’*-DDT and *p,p’*-DDE. Graphs A and B show the activity of *p,p’*-DDT in the 3 strains determined using the Malstat and SYBR Green 1 assays, respectively. Graphs C and D show the *p,p’*-DDE activity against the 3 strains also determined using the Malstat and SYBR Green 1 assays, respectively. The average of at least 8-30 determinations (2-10 biological repeats) determinations of each data point is shown.
This is the first study to our knowledge to assess the activity of \( p,p' \)-DDT and \( p,p' \)-DDE towards *Plasmodia* and in particular resistant *P. falciparum*. The comparable activity of \( p,p' \)-DDT and \( p,p' \)-DDE towards all three strains indicates that CQ resistance does not affect the activity of these compounds. This may mean that these chlorinated compounds do not enter the food vacuole as is the case with CQ or that they are not pumped out of the vacuole once entered. The extra chloride moiety on DDT also did not lead to a significant difference between the antiplasmodial activity of DDT and DDE. However, DDE is chemically more stable than DDT (Rogan and Chen 2005) and DDT seemed more prone to aggregation in stock solutions.

### 3.4 Conclusions

Based on the general experience of using both the Malstat and SYBR Green 1 assays for the *in vitro* drug assays, both assays proved to be simple and straightforward to use. The IC\(_{50}\)s obtained for all compounds tested were comparable for both assays (*Table 3.1*). The reagents used to make up the assay solutions were affordable. The specialised fluorescence spectrometer and specific black plates used for the SYBR Green 1 assay were, however, more costly than the assay plates and ordinary multi plate reader used for the Malstat method.

As part of the assay development, it was established that frequent sorbitol stress may influence the activity of CQ towards CQS D10 strains. Genetic studies to check for the presence and expression of the resistance gene(s) in the D10r parasites are, however, set for the future.

In this study, we were able to successfully determine the *in vitro* antimalarial activities of \( p,p' \)-DDT and \( p,p' \)-DDE for the first time against *P. falciparum* CQS D10 and CQR Dd2 strains. Comparable activity of ±14-38 \( \mu \text{M} \) (5-12 ppm or \( \mu \text{g/mL} \)) was observed for the three strains, which indicates that CQ resistance does not influence the activity of \( p,p' \)-DDT and \( p,p' \)-DDE towards *P. falciparum strains*. Individuals that are highly exposed to DDT may have blood circulating levels of DDT and/or its breakdown product DDE of between 103 ± 85 ppb (Bouwmann *et al.*, 1991). It has been shown for the first time, that based on the IC\(_{50}\)s of 14-38 \( \mu \text{M} \) (5-12 ppm) for \( p,p' \)-DDT (normally used to kill the mosquito vector) and \( p,p' \)-DDE against a very high parasitemia of 2\%, these individuals could actually be protected from *P. falciparum* malaria infections, which normally entails 10-100\( \times \) lower parasitemia. The effect or influence of
persistent low level exposure of DDT and/or DDE detected in blood on the activity of CQ in the CQS D10 parasites will therefore also be discussed in Chapter 4.

These results take us a step closer to determining the influence of these chlorinated compounds on the resistance or sensitivity of *P. falciparum* towards CQ. It is therefore important to understand the *in vitro* chemotherapeutic interactions between CQ and DDT and/or DDE during malaria infection and whether or not there is a link between these compounds to CQ resistance. This will be addressed in Chapter 4 through drug combination assays.

Assessing whether other insecticides also have similar activities would also assist in determining if exposure to the insecticides may have an influence on antimalarial drug resistance or sensitivity. *In vivo* mice models (*P. berghei*) could also be used in future studies to assess the effects of DDT, DDE and other pesticides on the *in vivo* CQ efficacy. However, this study focuses on the understanding the mode of activity of DDT and DDE on the malaria parasite in infected human erythrocytes, as little information is known at present. We will report on the effect of exposure of cultures to DDE, and DDT/DDE in combination with CQ on the activity of CQ against CQS and COR strains in Chapter 4.

### 3.5 References


CHAPTER 4

In vitro antimalarial drug interactions between chloroquine and the insecticide p,p’-DDT and its breakdown product p,p’-DDE

4.1 Introduction

The major aspects of the Global Malaria Eradication Program (GMEP) launched by the World Health Organisation (WHO) in 1955 involved the use of chloroquine (CQ) as an antimalarial drug and dichloro-diphenyl-trichloroethane (DDT) as an insecticide for vector control (Hay et al., 2004). This combination led to major successes in the eradication of malaria in some parts of the world especially the developed countries that had sufficient resources to make the program a success (Petersen et al., 2011). Drug and insecticide resistance, however, emerged resulting in a spike in malaria cases and deaths (Najera et al., 2011; Trape 2001; and Clyde and Shute 1957).

CQ resistance emerged as a result of mutations in the Plasmodium falciparum CQ resistance transporter (PfCRT) gene, which lies on the membrane of the parasite’s food vacuole, spreading to different parts of the world (Hayton and Su 2008). The “pump” acts by expelling the drug(s) from the digestive vacuole, thus preventing the formation of CQ-heme complexes and eventual toxic build up of heme in the vacuole which is proposed to aid in the killing of the parasite (Amina et al., 2010). The emergence of multidrug-resistant P. falciparum prompted the need to use combinations of antimalarial drugs, preferably those with different modes of action, to either increase drug efficacy or prevent/slow antimalarial drug resistance (White 1998). The P. falciparum multidrug resistance-1 (PfMDR-1) protein has also been linked to CQ resistance in Africa, and a functional relationship between PfCRT and PfMDR-1 proteins has been suggested (Hastings 2006; Barnes et al., 1992; Osman et al., 2007). Several mutations such as the K76T mutation in PfCRT, as further described in Section 1.3.2.2 of Chapter 1, lead to CQ resistance (Martin and Kirk 2004). The resultant multiple resistance profiles of some of the mutated transporters indicated that they can accommodate a broad spectrum of organic molecules (Amina et al., 2010).

Many of the compounds accommodated by the PfCRT and PfMDR-1 proteins contain a phenyl-chloride moiety. CQ, like DDT and its major metabolite dichloro-diphenyl-dichloroethylene
(DDE) contain such moieties, with the latter containing two each, and can possibly be accommodated by the transporters thus influencing CQ resistance. There, however, has been no study to our knowledge that has been published to determine the effects and/or subsequently the mechanism of action of DDT and DDE on erythrocytic stages of \textit{P. falciparum} or any other malaria strains.

The lipophylicity of DDT and DDE dictates that they associate with membranes, lipids, and hydrophobic proteins in biological media. To influence the malaria parasite directly, the DDT and/or DDE must reach the intra-erythrocytic malaria parasite. Again, there are, however, no published studies describing the interactions of CQ and DDT or DDE against the intra-erythrocytic malaria parasite. However, in a recent study a synthetic 2-hydroxydiphenyl ether with antimicrobial properties found in several detergents named triclosan, was found to have \textit{in vitro} and \textit{in vivo} antimalarial activity against blood stage \textit{P. falciparum} CQ sensitive and CQ resistant parasites (Surolia and Surolia, 2001; Mishra \textit{et al}., 2007). Triclosan shares some structural properties with DDT and inhibits the \textit{Fabl} enzyme involved in the conversion of acyl carrier protein, crotonyl-ACP, into butyryl-ACP in the \textit{P. falciparum} fatty acid biosynthesis II (FAS II) pathway. This pathway, which is not found in humans, is seen as a potentially attractive target for antimalarial drugs (Surolia and Surolia, 2001; Mishra \textit{et al}., 2007). The structural similarity (\textit{Figure 4.1}) between triclosan, DDT and DDE may provide a possible clue to the mechanism of action of DDT and DDE against the intra-erythrocytic malaria parasite.

The World Health Organisation recommends a combination of drugs that act on different parasite targets, and have favourable pharmacokinetic profiles for malaria treatment (He \textit{et al}., 2010). Despite the controversial association of DDT and its metabolites to adverse human health and environmental effects due to their long elimination half-lives (Turusov \textit{et al}., 2002), this study will attempt to elucidate the positive and negative influences of residual levels of the compounds in malaria control and may assist in justifying the reintroduction of the insecticide in malaria-endemic parts of sub-Saharan Africa. We will present our investigation on the \textit{in vitro} interactions of DDT and DDE with CQ against CQ sensitive D10 and CQ resistant Dd2 \textit{P. falciparum} strains and the influence of the malaria parasite on the distribution of DDT and DDE in \textit{P. falciparum} malaria cultures.
4.2 Methods and materials

4.2.1 Antimalarial drugs in this study

Chloroquine diphosphate salt (CQ, Mr 515.87), \( p,p' \)-DDT (Mr 354.49), \( p,p' \)-DDE (Mr 318.03) purchased from Sigma-Aldrich (Steinheim, Germany) were used for this study. Initial stock solutions of each of the compounds were prepared as described in Section 3.2.1 of Chapter 3 for the CQS D10 and CQR Dd2 strains.

4.2.2 In vitro \textit{P. falciparum} cell culturing

Laboratory grown \textit{P. falciparum} CQS D10 and CQR Dd2 were maintained in continuous culture that was enriched, synchronised and stored as described in Sections 3.2.2 to 3.2.6 of Chapter 3.

4.2.3 Antimalarial activity of CQ in combination with \( p,p' \)-DDT and \( p,p' \)-DDE

To determine the \textit{in vitro} drug-drug interactions of the compounds in the parasites, the activities of CQ in combination with \( p,p' \)-DDT or \( p,p' \)-DDE towards the CQS D10 and CQR Dd2 parasites were determined. A technique adapted from Chawira and Warhurst (1987) in which fixed ratios of predetermined concentrations required to inhibit parasite growth by 50\% (IC\textsubscript{50}) was used to determine the interaction of two drugs.

The combination assay was done using 2\% hematocrit, 2\% parasitemia cultures as described in Section 3.2.7 of Chapter 3. The drug combination solutions were serially diluted in RPMI media.
in pyrolysed glass vials cleaned as described in Section 2.2.2 of Chapter 2, using Hamilton glass syringes for volume measurement the previous day, and were stored at 4°C until required on the next day for the assays.

Using mass:mass ratios, combinations of CQ:p,p'-DDT and CQ:p,p'-DDE were prepared for the CQS and CQR parasite strains. The ratios of CQ to p,p'-DDT or p,p'-DDE were in ng/mL (nM or ppb) to µg/mL (µM or ppm) respectively. Therefore, from a highest concentration of 100 ng/mL CQ and 100 µg/mL p,p'-DDT or p,p'-DDE in the dilution range, the mass:mass drug combination ratios were 1:0, 0.075:25, 0.050:50, 0.025:75 and 0:1 respectively for CQS parasites (D10 strain). The highest concentrations in the dilution range for the CQR parasites (Dd2 strain) were 500 ng/L CQ and 100 µg/mL p,p'-DDT or p,p'-DDE, therefore the combination ratios were 1:0, 0.375:25, 0.250:50, 0.125:75, and 0:1 respectively. The total volume for each of the combined drug solutions prepared was 100 µL.

Eight serial doubling dilutions were prepared with the highest concentrations of the series being those indicated in the combination ratios. Separate solutions of each of the drugs (CQ, p,p'-DDT and p,p'-DDE) were used as controls for the experiment. GS at 20 µg/mL and drug solvents were used to determine the assay background and parasite growth respectively as described in Section 3.2.7 of Chapter 3.

4.2.4 In vitro parasite growth inhibition assays
To determine the parasite viability (Sections 3.2.7, Chapter 3) of each of the two strains (D10 and Dd2) after exposure of the parasites to the different drug combinations of CQ:p,p'-DDT and CQ:p,p'-DDE, dose response assays using the Malstat and SYBR Green 1 assays as described in Section 3.2.7.1 and 3.2.7.2 respectively of Chapter 3 were done.

4.2.5 Data analysis of in vitro drug–drug interactions
IC50 values were determined for all three ratios as well as for the drugs independently using both assays. Isobolograms were then constructed using Graphpad Prism® 4.0 (GraphPad Software Inc.) based on the fractional inhibition concentrations (FICs) calculated to present the mean IC50 values of the drug combinations as ratios, as described in Sections 4.2.8 and 4.2.9. At least three
technical repeats and three biological repeats were performed for each combination experiment for statistical purposes and to check the reproducibility of results.

4.2.6 Determination of fractional inhibition concentrations

Two fractional inhibition concentration (FIC) values, one for CQ and the other for \( p,p' \)-DDT or \( p,p' \)-DDE, were calculated separately for each of the five drug combination ratios by using the 50% inhibitory concentrations (IC\(_{50}\)s) of each of the two combined drugs.

The FIC for each drug combination was calculated using

\[
FIC_{\text{drug in combination}} = \frac{IC_{50}^{CQ} + DDx(\text{drug in combination})}{IC_{50}(\text{drug alone})}
\]

In Equations 4.1 (and 4.2 below), DDx represents \( p,p' \)-DDT or \( p,p' \)-DDE. The FIC of CQ or DDx is calculated as the ratio of the IC\(_{50}\) of CQ in combination with \( p,p' \)-DDT/\( p,p' \)-DDE to the IC\(_{50}\) of CQ or DDx alone that gives the same effect. The FICs of CQ (FIC\(_{CQ}\)) and \( p,p' \)-DDT or \( p,p' \)-DDE (FIC\(_{DDx}\)) were calculated for each combination, and isobolograms were plotted as described by Bell (2005).

Adapted from Odds (2003), the FIC\(_{\text{index}}\) was calculated as the sum of FICs of CQ and \( p,p' \)-DDT and/or \( p,p' \)-DDE using

\[
FIC_{\text{index}} = FIC_{CQ} + FIC_{DDx}
\]

FIC\(_{\text{index}}\) was used to determine the correlation between CQ and \( p,p' \)-DDT/\( p,p' \)-DDE so as to classify the combinations as either being synergistic, antagonistic or additive, with FIC\(_{\text{index}}\) values less than 1, more than 1 or equal to 1, respectively (Bell 2005; Berenbaum, 1978). For conservative interpretation, the cut off values were FIC\(_{\text{index}}\) \( \leq 0.5 \) for absolute synergism, 1\( > FIC_{\text{index}} > 0.5 \) for slight synergism, FIC\(_{\text{index}} = 1 \) for additive activity, non-interactive to slight/moderate antagonism at 1\( < FIC_{\text{index}} < 4 \), or absolute antagonism with FIC\(_{\text{index}} \geq 4 \) (Odds 2003; Gupta \textit{et al.}, 2002; Bell 2005).

4.2.7 Isobologram construction

With the FIC\(_{\text{index}}\) data giving a numerical indication of the drug combination interactions, graphical representation in the form of isobolograms was also used to interpret the possible
different interactions of the drugs (Odds, 2003; Gupta et al., 2002; Bell, 2005). Data points of each combination ratio in relation to the FICs were plotted and examined.

As described by Bell (2005), the shapes of the lines plotted from the FIC data were interpreted as either being synergistic (concave; points below the dotted line), additive to non-interactive (linear; points on or near the dotted line) or antagonistic (convex; or points above the dotted line) based on the combinations of the two drugs as described in Figure 4.3. The strength of either synergism or antagonism would be indicated by how much the respective curves deviate from the line of additivity.

4.2.8 Assessment of p,p’-DDE exposure on CQ resistance or sensitivity
CQS D10 parasites cultured as described in Section 3.2.3 of Chapter 3 were grown for nine days using modified culture media containing 10 ppm p,p’-DDE. Cultures (10 mL) were maintained on a daily basis and a small sample (200 μL) was taken each day for analysis. Unmodified media was then used to culture the parasites to a parasitemia ≥ 10%, and the sensitivity of the parasites against CQ, p,p’-DDT and p,p’-DDE was then assessed as previously described in Sections 3.2.5, 3.2.7.1 and 3.2.7.2 of Chapter 3 using parasite counts, the Malstat and SYBR Green 1 assays respectively.

4.2.9 Compartment distribution of p,p’-DDT and p,p’-DDE in P. falciparum cultures
p,p’-DDT and p,p’-DDE distribution in the malaria cultures was determined to assess how much of residual p,p’-DDT and/or p,p’-DDE would enter the different compartments (the RPMI media and packed erythrocytes) over time. The distribution between CQS D10 parasitised packed erythrocytes and RPMI media was also assessed to determine whether the parasites would have an influence on the distribution of the drugs between the compartments.

The biological media (packed erythrocytes plus RPMI media) was spiked with 5ppm p,p’-DDT and/or p,p’-DDE. The spiked samples were then incubated at 37 °C at different time intervals (0, 1, 2, 4, 8, 16 and 24 hours) before being extracted using the method described in Sections 2.2.5 of Chapter 2. Samples were prepared at least in duplicate for statistical purposes.

After the incubation period, the biological media was then separated into two compartments of packed uninfected erythrocytes and RPMI media by being centrifuged at 750×g for 3 minutes.
Each compartment was then extracted as described above. Residual levels of \( p,p' \)-DDT and \( p,p' \)-DDE in each compartment were then determined using GC-MS and HPLC-UV analysis methods as described previously in Sections 2.2.7, 2.2.8 and 2.2.9 of Chapter 2. Unspiked packed erythrocytes and RPMI media mixtures were used as the experimental controls, and were extracted (Sections 2.2.6 of Chapter 2) and analysed in the same manner as described for the \( p,p' \)-DDT and \( p,p' \)-DDE spiked samples.

The CQS D10 cultures (\( \geq 10\% \) parasitemia in ring stage) spiked with 5 ppm \( p,p' \)-DDT and \( p,p' \)-DDE were separated into parasitised packed erythrocytes and RPMI media by centrifuging at 750\( \times g \) for 3 minutes. Residual levels of the compounds were extracted and analysed in both the RPMI media and the parasitised erythrocytes using GC-MS and HPLC-UV analysis methods after exposure to the compounds at different time intervals and extraction (methodology described in Chapter 2). Unspiked D10 parasitised erythrocytes and RPMI media mixtures were used as the experimental controls.

4.3 Results and Discussion

4.3.1 In vitro antimalarial activity of CQ in combination with \( p,p' \)-DDT and \( p,p' \)-DDE

As part of assessing the influence of DDT and DDE exposure on chloroquine resistance, the in vitro antimalarial activities of CQ: \( p,p' \)-DDT and CQ: \( p,p' \)-DDE drug combinations were determined. The activity of the compounds in combination would in turn lead to determination of the in vitro drug-drug interactions of the compounds in the CQS and CQR parasites of \( P. falciparum \) and possibly provide a clue towards the mechanism of action of especially DDT and DDE since several drug combination studies are used for elucidation of drug mode of action (Bell 2005). The fixed ratio technique adapted from Chawira and Warhurst (1987) and the checkerboard method described by Odds (2003) have been widely used to assess antimalarial drug interactions (Fivelman et al., 2004; Bell 2005). Both methods depend on the predetermination of the IC\(_{50}\)s of the combination drugs.

The IC\(_{50}\) values of the drug combinations were determined with normal dose response assays as described in Chapter 3. Representative dose responses with the CQ sensitive strain, \( P. \)
*falciparum* D10 as the combination drug target are shown in Figure 4.2. Similar trends for both strains were observed with the Malstat and SYBR Green 1 assays (results not shown).

**Figure 4.2** Representative dose response graphs of the different drug combinations in terms of the CQ against CQS *Plasmodium falciparum* D10 parasites. Assays were developed using the Malstat assay and each data point represents the mean of triplicate determinations with standard error of the mean (SEM).

FIC values were calculated from the IC\textsubscript{50}s obtained with the different drug combinations directed towards the sensitive D10 and resistant Dd2 *P. falciparum* parasite strains. We constructed isobolograms (*Figure 4.3*) in order to assess the effect that the drugs have on each other’s activity. Slight synergistic trends (concave shaped graph below the line of additivity) were observed with both *p,p*-DDT and *p,p*-DDE in combination with CQ towards the CQS D10 strain (*Figure 4.3*). However, the complete opposite trends were found for both *p,p*-DDT and *p,p*-DDE in combination with CQ towards the CQR Dd2 strain which showed moderate
antagonistic trends (convex shaped graph above the line of additivity) with both compounds (Bell 2005; Berenbaum 1978).

![Graph showing isobolograms](image)

**Figure 4.3** Isobolograms depicting the *in vitro* drug interaction between CQ and *p,p*'-DDT (A) or CQ and *p,p*'-DDE (B) towards *P. falciparum* CQS D10 and CQR Dd2 strains. The FIC values were derived from at least 3 independent experiments done at least in triplicate for each drug combination concentration in each set and determined using the Malstat assay. SEM is shown for each averaged data point.

To further investigate the effect of the drug combinations in the above finding, we calculated FIC*indexes using data from both the Malstat and SYBR Green 1 assays (Table 4.1). The calculated FIC*index parameters from both assays correlated well with each other and with our previous assessment using the isobolograms. When the results are not clear cut and the FIC*index values are either just below 1 or just above 1, this leaves some doubt as to whether the drug combinations are significantly synergistic or antagonistic. Therefore we opted to use more conservative cut off values with FIC*index ≤ 0.5 for absolute synergism, slight synergism at 1 > FIC*index > 0.5, additive activity at FIC*index = 1, non-interactive to slight/moderate antagonism at 1 < FIC*index< 4 (adapted from Odds 2003; Gupta *et al.*, 2002; Bell 2005). Using these FIC index limits, our FIC*index results show a slight/moderate antagonism between CQ and *p,p*'-DDT and *p,p*'-DDE for the CQ resistant Dd2 strain and slight synergism for CQ sensitive D10 strain.

The synergistic effect observed in the D10 strain may have been as a result of increased effectiveness of CQ and *p,p*'-DDT or *p,p*'-DDE collectively working in combination at different
target sites in the malaria cultures. CQ targets the intra-erythrocytic parasite directly, specifically via the hemozoin polymerization in the food vacuole (O'Neill et al., 2006), while the target(s) of \( p,p' \)-DDT and \( p,p' \)-DDE are yet to be elucidated. However, the antagonism observed with the Dd2 parasites, may suggest that \( p,p' \)-DDT and \( p,p' \)-DDE have an effect on CQ resistance or the action of CQ on a target other than hemozoin polymerization.

Table 4.1 Summary of the FICs and calculated FIC index to describe the *in vitro* drug interaction between CQ and \( p,p' \)-DDT or \( p,p' \)-DDE in different combinations against *P. falciparum* D10 and Dd2 parasite strains. The top parameters in each row were determined using the Malstat assay and the bottom value with the SYBR Green 1 assay. N depicts the number of biological repeats, and DDx depicts \( p,p' \)-DDT or \( p,p' \)-DDE. Each biological repeat was done using at least triplicate technical repeats.

<table>
<thead>
<tr>
<th>Drug Combination ratio CQ:DDx</th>
<th>CQ FIC</th>
<th>DDT FIC</th>
<th>CQ:DDT FIC(_{\text{index}}) (n)</th>
<th>CQ FIC</th>
<th>DDE FIC</th>
<th>CQ:DDE FIC(_{\text{index}})</th>
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<tr>
<td><strong>P. falciparum D10 (CQ sensitive)</strong></td>
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4.3.2 *Assessment of DDT and DDE exposure on CQ resistance or sensitivity*

As DDT and DDE may reside for prolonged periods in the blood they may, apart from the antagonistic or synergistic actions on CQ, have an effect on the malaria parasite. We compared parasite sensitivity towards CQ for parasites adapted in blood from three different donors with different levels of residual blood DDT and/or DDE as determined by the HPLC-UV and GC-MS.
methods discussed in Chapter 2. Donor 1 may have 1-2 ppm whilst Donor 2 had trace amounts of circulating DDT and/or DDE (lower than the GC-MS and HPLC-UV instrument detection limits) (Table 2.6 of Chapter 2). Donor 3 CQ was used as the control group since no DDT or DDE was detected in the blood.

Although the determined IC50 values of CQ toward the CQS D10 strain grown in the blood of the three donors was comparable (Figure 4.4), it was unclear whether the difference in dose response curves concerning their tops and the shifts to the right was due to a donor effect or resistance/antagonism due to low level exposure to DDT and/or DDE, or are a result of both. The donor effect for example could entail differences in the blood lipid content, age of the respective red blood cells or other cellular differences and residual drugs that may interfere with CQ action or parasite growth.

A comparison of inhibition at 48 nM (25 ng/mL) CQ showed significant difference (P < 0.05) between donors 1 and 2. At this concentration, only about 50 % growth inhibition of the parasite was achieved with donor 1 blood compared to slightly over 70 % parasite growth inhibition with donor 2 blood as shown in Figure 4.5.

To determine if there is a longer term effect of p,p′-DDE, the major breakdown product of p,p′-DDT, on the parasite’s response to CQ, CQS D10 parasites were grown for nine days in the three
different sets of donated blood and maintained in modified culturing media containing 10 ppm \( p,p' \)-DDE. As seen above, donor blood could affect CQ sensitivity (Figures 4.4 and 4.5), as well as have a major effect on the growth rate of the parasite (Figure 4.6).

**Figure 4.5** Comparison of the difference in 48 nM (25 ng/mL) CQ inhibitions towards *P. falciparum* D10 parasites between blood from 3 donor sets with differing levels of residual DDT and/or DDE, determined using the Malstat Assay.

From our studies it was clear that the parasites adapted better in the blood of some donors than others, with donor 3>2>1 (Figure 4.6). The rapid fall in parasitemia within five days in the blood of donor 3 indicate a rapid selective killing of fast growing parasites. Although the initial parasitemia differed for the different blood donors, approximately 3% of the parasites survived in each case. Therefore, regardless of the donor blood, growing the parasites in the presence of 10 ppm \( p,p' \)-DDE led to the emergence of persistent slow growing cultures (Figure 4.6). This may partly explain the above results (Figure 4.5) with parasites grown in donor 1 blood being less sensitive. The parasites that survived the \( p,p' \)-DDE exposure were re-cultured to roughly 10% parasitemia in the absence of \( p,p' \)-DDE in the blood of donor 3. The CQ, \( p,p' \)-DDT and \( p,p' \)-DDE sensitivity of the parasites was assessed as previously described in Chapter 3. Based on the IC\(_{50}\)s obtained and compared to those previously obtained before exposure of the CQS D10 parasites to the 10 ppm \( p,p' \)-DDE (Chapter 3), there was no significant change in the activities of CQ, \( p,p' \)-DDT and \( p,p' \)-DDE against the re-cultivated parasites (Figure 4.7).
Figure 4.6 Survival of *P. falciparum* D10 grown in the presence of 10 ppm *p*,*p*' -DDE over 9 days of exposure.

Figure 4.7 CQ induced dose response of *P. falciparum* CQS D10 infected cells that survived the nine day exposure to 10 ppm *p*,*p*' -DDE. Percentage growth inhibition induced by CQ (A), DDT (B) and DDE (C) was determined using the colorimetric based Malstat assay. The average of determinations of each data point from four technical and two biological repeats each are shown with SEM. IC$_{50}$s were calculated from the sigmoidal dose response curves generated from the different data points.
The activity of \(p,p'\)-DDT and \(p,p'\)-DDE in terms of IC\(_{50}\) values remained similar to those determined before exposure to 10 ppm \(p,p'\)-DDE (Figure 4.7). This suggested that persistent slow growing parasites that survive after exposure to DDT and/or DDE in the human host still remain sensitive to CQ, \(p,p'\)-DDT and \(p,p'\)-DDE at the same level of potency as would in an individual not exposed to DDT and/or DDE. These results indicated that the persistent parasites did not acquire resistance against any one of the three compounds during the nine day exposure to 10 ppm \(p,p'\)-DDE.

4.3.3 \(p,p'\)-DDT and \(p,p'\)-DDE distribution in \textit{P. falciparum} D10 cultures

As it was clear from our results on the combination of CQ with \(p,p'\)-DDT and/or \(p,p'\)-DDE that the compounds act slightly synergistic against the CQS D10 strain it was important to assess how much of residual \(p,p'\)-DDT and/or \(p,p'\)-DDE would enter into or remain in the different compartments (the RPMI media, erythrocytes and infected erythrocytes) over time. CQS D10 parasitised erythrocytes were used to assess if the presence of the parasites would at all influence the distribution of the drugs between the compartments. The distribution of the compounds between the compartments could give an indication if the compounds are pumped into or out of the parasite, trapped in the erythrocyte compartment and if it is a slow or fast equilibrium between compartments. In future studies in which we intend to determine how much of the DDT and/or DDE eventually enter the intra-erythrocytic parasite if any, and what concentration levels are present to either antagonise CQ in the resistant strains or work in synergy to fight malaria infection in CQS strains so as to enable effective dosage of CQ in the malaria infected patients.

The GC-MS and HPLC-UV analysis gave comparable trends in the distribution observed with both analysis methods (refer to Figures 4.8 and 4.9). There was, however, some discrepancy in that 5-20% higher levels of \(p,p'\)-DDT and \(p,p'\)-DDE was detected with GC-MS in the erythrocytic compartment corresponding with similar lower levels in the medium. This difference may be due to background interference in our HPLC method.

Our initial observation was that the 24 hour exposure period of the biological media containing infected erythrocytes to \(p,p'\)-DDT and \(p,p'\)-DDE allows enough time for the compounds to reach equimolar distribution between the different compartments (results not shown). This distribution
is probably due to binding of the hydrophobic compounds to the lipophylic membranes and proteins with hydrophobic pockets in the media or cytoplasm.

As is shown in Figures 4.8 and 4.9, there was a high initial erythrocyte association in the absence of infected erythrocytes and an almost equimolar distribution of the initial 5 ppm \( p,p' \)-DDT between the erythrocytes and RPMI medium after 16 hours. This gave a strong indication that a substantial amount of each of the compounds could eventually reach the intra-erythrocytic malaria parasite since at least 50% of the compound is associated with the erythrocyte compartment.

![Figure 4.8 Distribution of \( p,p' \)-DDT over 16 hours of exposure between erythrocytes (blood/infected blood) and RPMI media (media/infected media). The distribution profiles were determined by A) HPLC-UV and B) GC-MS analysis with 2-5 biological repeats for each time point and were shown with error bars indicating SEM.](image)

The slow equilibrium between the uninfected erythrocytes and media may have been due to the fact that after initial spiking of the biological media, \( p,p' \)-DDT and \( p,p' \)-DDE bound more to the surface lipids of the red blood cells then slowly equilibrated back into the RPMI media, possibly binding to albumin until almost equimolar levels were reached in both compartments.
The high initial erythrocyte association was even more pronounced in the presence of D10 infected erythrocytes indicating that the parasite infected erythrocytes may initially associate even better with \( p,p'\)-DDE and \( p,p'\)-DDT. The compartment equilibrium for both \( p,p'\)-DDT and \( p,p'\)-DDE was reached at a much faster rate in the cultures with \( \pm 10\% \) initial parasitemia (Figures 4.8 and 4.9). This may be due to the increased permeability of the infected erythrocyte membrane (Kirk 2001). However, the compartmental distribution of \( p,p'\)-DDT differed significantly from that of \( p,p'\)-DDE, which also reached equimolar distribution between the two compartments. The D10 parasites appear to cause trapping of \( p,p'\)-DDT in the erythrocyte compartment with very little of this compound being release from the erythrocytic compartment. We observed a 30-50\% higher amount of \( p,p'\)-DDT that was retained in the erythrocytic fraction of the D10 infected cultures.

**Figure 4.9** Distribution of \( p,p'\)-DDE over 16 hours of exposure between erythrocytes (blood/infected blood) and RPMI media (media/infected media). The distribution profiles were determined by A) HPLC-UV and B) GC-MS analysis with 2-5 biological repeats for each time point and were shown with error bars indicating SEM.
4.4 Conclusions

When the potency of two or more drugs combined in an assay of antimalarial activity is either higher or lower than would have been expected from the individual activities, synergy and antagonism, respectively, are obtained. The drugs may or may not interact physically to produce these effects. However, usually it is the influence of the activity of the one drug that affects that of the other in the combination whilst acting on the respective target sites. The existence and strength of drug interactions may therefore provide information on what the individual drugs do in the cells (Bell 2005).

We found slight synergistic activity between CQ and \(p,p'\)-DDT or \(p,p'\)-DDE towards the CQ sensitive D10 strain and notable antagonism towards the CQ resistant Dd2 strain. It may be that the two compounds act antagonistically and enhance resistance indirectly by competing for the same target, possibly a non-vacuolar target, or by stimulation of the CQ transporter in the food vacuole. The synergy, however, may be due to different target sites of CQ and DDT or DDE allowing for effective killing of the parasites. The observation of reciprocal synergism of \(p,p'\)-DDT and \(p,p'\)-DDE with CQ against CQS D10 and antagonism against CQR Dd2 strain is highly significant and strongly indicates selection of CQ resistant strains in the presence of \(p,p'\)-DDT and \(p,p'\)-DDE. People who have low levels of circulating DDE and/or DDT could be at higher risk to contract CQR malaria.

On the rationale for malaria combination therapy White (1998), however, argues that although certain drug combinations show antagonism \textit{in vitro}, the effects are generally small and there is no evidence that this translates into a reduced \textit{in vivo} effect. Triclosan was shown to potently inhibit \textit{P. falciparum} \textit{in vitro} and in an \textit{in vivo} mouse model (Surolia and Surolia 2001). Since we hypothesise that DDT and DDE may share the same mechanism of action with triclosan because of the structural similarities, then they may also be equally potent when the drug combinations with CQ are introduced to an \textit{in vivo} model. However, it is important to first explore their mechanism(s) of action and compare it/them with that of triclosan in future studies.

Persistent growing parasites exposed to 31.4 µM (10 ppm) \(p,p'\)-DDE for nine days did not acquire resistance against CQ, \(p,p'\)-DDT or \(p,p'\)-DDE therefore this may be reflective of what can be expected in the \textit{in vivo} studies. Future work will, however, involve exposure of the D10
strain to 10 ppm DDT for the nine days or more, and of both DDT and DDE on CQR Dd2s. Resistance markers will then be assessed to determine if DDT and/or DDE have an influence on strain mutation or adaptation.

The distribution study gave a strong indication that DDT and/or DDE will eventually reach the intra-erythrocytic malaria parasites since some of the compounds had already reached the erythrocyte itself. The parasites were shown to trap DDT for longer than DDE before the compounds are distributed between the different compartments. Compartment distribution within the CQS cultures was done with the aim of eventually determining how much (if any) of DDT and DDE would eventually enter the malaria parasite itself. These future studies will give an indication of what concentration of DDT or DDE in the parasite could have an in vivo effect that may compromise the treatment of malaria patients previously exposed to DDT. This study will also be followed up in future work with assessment of how the CQR cultures influence the compartment distribution if at all. Optimised GC-MS and HPLC-UV methods used and developed in Chapter 2 will be used to determine the DDT and DDE levels in the parasites.

4.5 References


CHAPTER 5

Conclusions

The overall goal of this study was to assess if low levels of DDT and/or DDE, circulating in blood of people exposed to these insecticides, have activity on the asexual blood stages of *Plasmodium falciparum* and if this exposure could lead to the selection of *P. falciparum* strains resistant to chloroquine.

The initial determination of background levels of DDT and DDE in blood and malaria culturing media, to assess the contaminant variable, was successfully conducted with the development and optimisation of an HPLC-UV and two GC-MS analysis methods (*Chapter 2*). Good recoveries of the DDT and DDE were obtained from biological samples (blood and malaria culture RPMI media) and were consistently above 86% for both DDT and DDE as determined with our three analyses methods. Although an effective and reproducible HPLC-UV analysis method was successfully developed for the determination of high concentrations of DDT (up to 100 ppm) and DDE (up to 50 ppm) in the biological media, low concentrations could not be accurately determined as there was possible biological interference that could result in false higher levels detected in the blank biological samples compared to GC-MS methods. An optimised and more sensitive GC-MS method, however, proved to be more effective in determining lower concentrations (≤ 1 ppm) of DDT and DDE with LOD of 7.8 ppb and 78 ppt for DDT and DDE, respectively, compared to 78 ppb and 7.8 ppb, respectively, with the HPLC-UV method. DDT, however, disintegrated into DDE and DDD with this method, which was not observed with the HPLC-UV method, possibly due to high column temperatures.

We determined the effect of 2.2 to 282 and 314 µM (0.78 to 100 ppm) of *p,p’*-DDT and *p,p’*-DDE respectively, on the viability of CQS and CQR strains of *P. falciparum* (*Chapter 3*). We were able to achieve comparable results with two *in vitro* drug assays (Malstat and SYBR Green). *p,p’*-DDT and *p,p’*-DDE were tested at relatively high parasitemia of 2%, which relates to a serious malaria infection and they exhibited *in vitro* IC50s towards a 2% culture of *P. falciparum* CQS D10 and CQR Dd2 strains of between 5 to 12 ppm or ± 14 to 38 µM (*Chapter 3*).
This is the first study in which it has been illustrated that low levels of the insecticide DDT and its major metabolite, DDE, could offer protection against blood stages of *P. falciparum* malaria. Although it has been argued that *in vitro* studies do not always give a true indication or correlation of what to expect with *in vivo* studies (White 1998), it remains a possibility that this activity could translate to *in vivo* activity. Previously exposed individuals with low levels of DDT and/or DDE circulating in their bloodstream may actually be protected against malaria infection. Future studies would include the *in vivo* effects of DDT and DDE in a *P. berghei* mouse model.

The actual targets of DDT and DDE were not established and will be addressed in future studies. We, however, hypothesize that these compounds may have a similar target to an analogous compound, triclosan. Triclosan was shown to have antimalarial activity *in vivo* and *in vitro* by inhibiting enoyl-ACP reductase of *P. falciparum* (Surolia and Surolia, 2001). Triclosan, however, was not available to us at the time of conducting the experiments thus could not be used for comparative studies with DDT and DDE.

Further positive evidence for the protective role of DDT and DDE is described in *Chapter 4*. Drug combination studies showed that CQ with DDT and CQ with DDE worked slightly synergistically at different combination ratios in the killing of CQ sensitive D10 strain. Although exposure of the parasites to 10 ppm DDE for 9 days showed a possible selection for consistently slow growing parasites, this, however, did not significantly affect the sensitivity of CQ in parasite killing. These results show a possible positive impact of residual levels of DDT and DDE in the blood of malaria infected patients when treated with the conventional antimalarial drug CQ. However, we also found some disturbing results concerning the CQ resistant strain Dd2 in that antagonism was shown for both DDT and DDE in combination with CQ, indicating that DDT and DDE could assist in selecting CQ resistant strains. This latter finding may explain the sudden coincidental rise of CQ resistance after the DDT spraying was terminated in certain areas.

In order to further probe the influence of DDT and DDE on the malaria parasites, we assessed the distribution of the compounds between media and normal/infected erythrocytes (*Chapter 4*). The lipophylic nature of DDT and DDE would probably dictate that they associate more with
hydrophobic environments such as membranes, lipids, membrane proteins and proteins with hydrophobic pockets (i.e albumin), than aqueous media such as culture media and cytoplasm. In infected cultures it was observed that the D10 parasites enhanced the distribution equilibrium of DDT and DDE between the erythrocytes and RPMI media. DDE reached to almost equimolar levels after 8 hours exposure while this distribution was much slower in culture with normal erythrocytes. However, 30-50% more DDT than DDE remained in the infected erythrocyte fraction giving a strong indication that a major fraction of DDT and a large fraction of DDE may eventually end up in the intra-erythrocytic parasite. These results may partially explain the slight synergism that was found between DDT/DDE and CQ against the CQ sensitive D10 strain. In future studies we will assess the distribution of DDT and DDE using the CQ resistant strain Dd2, as well as other sensitive and resistant strains. This will hopefully assist in the elucidation of the antagonism between CQ and DDT/DDE towards the CQ resistant Dd2 strain. Using the appropriately optimised analysis methods (Chapter 2) as well as radioactively labelled compounds, we also intend to determine how much of the DDT and/or DDE eventually end up in the parasite itself in relation with CQ. We will also include in this study an investigation on the influence of DDE and DDT on the proteins such as the PfCRT and PfMDR-1 (refer to Chapter 1), that are involved in the resistance to CQ and other analogous antimalarial drugs.

Overall, this study opens new doors for future research on malaria control strategies and drug discovery. These results highlighting the “good and bad” of DDT in terms of the malaria parasite may aid in the development of optimal antimalarial treatment policies in high prevalence malaria countries in which policies against the use of DDT are pushed forward and questions on whether the benefits of continued DDT use outweigh its detrimental effects to the environment and to human health arise.

As antimalarial drug and insecticide resistance remain obstacles in controlling malaria (Guerin et al., 2002), more studies need to be done and possible drug combinations that have different target sites explored, to slow down the development of antimalarial drug resistance (White 2008). The correlation between DDT and/or DDE and resistance to other antimalarial drugs needs to be investigated as there may also be similar interactions as those with CQ and DDT and DDE. The future studies also need to address the influence of structurally similar aromatic
compounds, organochlorine or organophosphate insecticides used for indoor residual spraying, and pyrethroids used in insecticide treated bed nets that may enter an infected individual’s blood on the different classes of conventional antimalarial prophylactics.

References

