A VALIDATED ANALYTICAL METHOD FOR THE DETERMINATION OF LUMEFANTRINE IN SELECTED TIME MODULATED DRIED BLOOD SPOT SAMPLES FROM MALARIA PATIENTS IN BOTSWANA

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A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN ANALYTICAL CHEMISTRY.

March, 2020

CERTIFICATION

The signatories below certify that they have read and hereby recommend for acceptance by the department of Chemistry, University of Botswana, a dissertation entitled **"A VALIDATED ANALYTICAL METHOD FOR THE DETERMINATION OF LUMEFANTRINE FROM SELECTED MALARIA INFECTED HUMAN SAMPLES FROM BOTSWANA COLLECTED AS DRY BLOOD SPOTS"** in accordance with the requirements of Masters of Science degree in Chemistry at University of Botswana.

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Date: ……. /……. /2020	**Date: ……/…….. /2020**	**Date: ……. /…….. /2020**

DECLARATION AND COPYRIGHT

I hereby declare that the dissertation submitted for the fulfilment of Masters of Science in Chemistry to the University of Botswana, Gaborone is my original work and has not been submitted to any other institution and any work quoted is indicated and acknowledged by means of comprehensive list of references.

……..

BLONDIE OABILE BROOKS

Date: ……/……. /2020

DEDICATION

This research is loving and wholeheartedly dedicated to my mother; Lily Brooks, my two sister Rose and Oratile as well as my respective relatives for their support.

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My first and far most acknowledgement goes to my Lord and savior Jesus Christ for strength, wisdom and knowledge He granted me to complete this research. The guidance and leadership of the Holy Spirit through trials and test of this research work is acknowledged without you nothing can be accomplished.

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ABSTRACT

A simple liquid chromatography- diode array detector (LC-DAD) method for the determination of lumefantrine in whole blood collected in dried blood spot (DBS) filters was developed and validated. The validation was done using the United States Food and Drug Administration (USFDA) guidelines. Sample preparation was done using solid liquid extraction (SLE) followed by separation using high performance liquid chromatography (HPLC) and diode array detection (DAD). Separation was done at 25ºC using an XTerra C18 column with dimensions of 50 mm x 4.6 mm x 5µm (length x internal diameter x particle size) and a binary solvent system of acetonitrile and water adjusted to pH of 2.3 with formic acid as the mobile phase. The mobile phase was pumped at a flow rate of 0.570 mL/min using a gradient elution program. The analysis time was 2 minutes and the calibration curve obtained was linear over the concentration range of 1-8 µg/mL with a correlation coefficient (R^2) of 0.9980. The limit of detection (LOD) and the lower limit of quantification (LLOQ) were 0.4 and 0.8 µg/mL respectively. The extraction efficiency estimated as percent recovery was greater than 60 %. Both the intra and inter-day precision of this method were < ±15% as prescribed by the USFDA guidelines. The method was successfully applied for the quantification of lumefantrine in time modulated dried blood spot samples, previously collected from patients on malarial treatment with the artemisinin/ lumefantrine combination therapy.

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LIST OF UNCOMMON ABBREVIATION AND SYMBOLS

ACTs	Artemisinin Combination Therapies
AL	artemether-Lumefantrine
Caf	Caffeine
CQ	chloroquine
CTs	combination therapies
CV	coefficient of variation
DAD	diode array detector
DBL	Desbutyl-lumefantrine
DBS	Dried Blood Spot
GC	Gas chromatography
HPLC	High Performance Liquid Chromatography
IDLs	Instrument detection limit
IS	Internal standard
ITNs	Insecticides- treated nets
LC-DAD	Liquid chromatography-diode array detector
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LC-UV	Liquid chromatography-ultraviolet
LLOQ	Lower limit of quantification

LOD	Limit of detection
LUM	Lumefantrine
m/z	mass to charge ratio
MDLs	Method detection limit
QCs	quality control
RDT	Rapid Diagnosis Tests
SD	standard deviation
SE	standard error
SLE	Solid-Liquid extraction
SP	sulphamethoxsazol-Pyremethamin
ULOQ	Upper limit of quantification
UV-Vis	Ultraviolet Visible
WHO	World Health Organization

CHAPTER ONE

1.0 INTRODUCTION

1.1 Overview of Malaria in Botswana

Botswana is a landlocked country, sharing boarders with four countries: South Africa, Zambia, Namibia and Zimbabwe. Malaria transmission in Botswana is said to be highly seasonal thus related to the distribution and level of rainfall occurring between December and April and this is more predominant in northern and northeastern parts (Department of Public Health, Botswana, 2015; Chihanga et al, 2016; World Health Organization, 2010). According to the Department of Health, Botswana, (2015) and Motlaleng *et al* (2018), the transmission is most intense in Chobe, Okavango and Ngami and is caused by plasmodium *falciparum* which constitute 98% of all malaria cases and 2% shared by *malariae* and *ovale* (Ministry of Health, Botswana, 2007). The World Health Organization (WHO) reported that nearly 28% of the population located in the northern parts of Botswana is at high risk, 37% which is located at the central is at lower risk and the remaining percent which is in the South is free of malaria. Still in Botswana, confirmed malaria cases declined by 71% from 3362 during 2000-2005 to 951 cases in 2009 (World Health Organization., 2010). In the same period, malaria related deaths were also reported to have declined from 21 to 6 deaths (World Health Organization., 2010). Figure 1.1 below shows the geographical distribution of confirmed malaria cases in Botswana adapted from (World Health Organization., 2010).

Figure 1.1. Map of Botswana showing confirmed cases of malaria per 1000 population ((World Health Organization., 2015).

1.2 Antimalarial Drugs

In general there are six classes of antimalarial drugs namely; "aminoquino-lines, arylaminoalcohols, artemisinins, antifolates, antibiotics and inhibitors of the respiratory chain" (Sharma *et al*., 2013), all of them possessing a specific chemical structure for antimalarial activity. After the emergence and spread of *P. falciparum* multi-drug resistant, combination therapy (CT) as a first-line drug treatment for uncomplicated malaria was introduced, and this has opened a new horizon in the fight against malaria, with extraordinary results. Generally this combination therapy involves artemisinin in combination with long acting antimalarial drugs (Zuma *et al*., 2016). In agreement with Zuma; Bloland and co-workers recounted that "the effect of combination therapy is boosted by the inclusion of artemisinin derivatives because it decreases parasite density more rapidly than other drugs". This offered a starting point for development of current drugs to fight malaria. There are common and important factors that have made this advancement fruitful. These are preservation and maintenance of antimalarial activity (related to the structure), improvement

of drug efficacy by pharmaceutical companies (Zuma *et al*., 2016), as well as strengthening the life cycle stage effect, molecular target and half-life of malaria drugs (Daily., 2006).

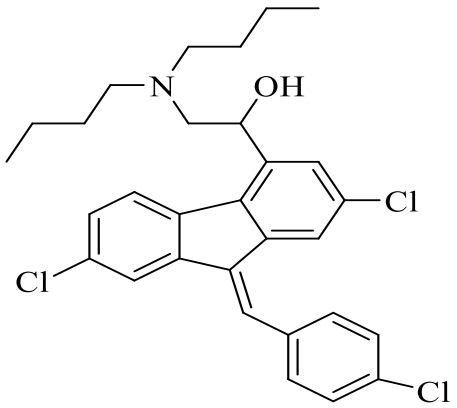
The fundamental factor of all of them is preservation of important structures responsible for antimalarial activity (in short, the chemistry) and as mentioned, the current focus is with artemisinin in combination with other antimalarial drugs. Thus, there is preservation of antimalarial activity structures. Before Combination therapy (CT), the history showed that chloroquine (CQ) and other drugs were used as first-line monotherapy treatment, with chloroquine (QC) being the most used. This was so, before its effectiveness was exposed by parasite resistance due to its frequent administration. It was widely used due to its affordability and its effectiveness, and this prompted for new target and affordable drugs to be developed due to resistance of almost all effective antimalarial drugs (Jones *et al*., 2015). The development of a new and effective drug focused on the artemisinin combination therapies (ACTs).

It is believed that a short half-life antimalarial drug, lowers the development of resistance risks when compared with a long half-life one (Bloland *et al*., 2000), Recent reports made, agrees and established that, to outstand the resistance of parasite for monotherapy, a combined therapy treatment especially using artemisinin (short half live) and other antimalarial drugs is a key and should be applied. Recommended by the WHO, the administration of these CTs reduces the probability of resistance developing simultaneously to two chemotherapeutic agents with independent mechanisms of action. These also reduces the overall malaria transmission rates, particularly when using artemisinin in combination with other antimalarial drugs (Bloland *et al*., 2000). Current combinations recommended by WHO are Artemether-Lumefantrine (also known as coartem), artesunate-amodiaquine, artesunate-mefloquine and artesunate-Sulfadoxide-pyrimethamine (SP) etc. These combinations are said to be a fixed dose. According to Daily it

was recommended that Artemether-Lumefantrine combination be used as standard treatment in Africa, but there is no restriction since these combinations can vary from one country to the another (Daily, 2006). This was because of the geographical location which is said to affect genetic codes as well as the type of the mosquito parasite. It is these variations that bring about the need to study these drugs in patients as a way of monitoring them and studying their bioavailability.

1.2.1 Lumefantrine

Lumefantrine, also known as benflumetol, was synthesized in china around 1970s. It is currently used as a long-acting antimalarial drug and administered in combination with the artemisinin family. It consists of short-acting mechanism to improve the efficacy (Pingale & Mangaonkar., 2013). This drug is said to be absorbed and cleared slowly with an elimination half-life between 3 and 4 days in malaria patients and six days in healthy volunteers (Djimdé and Lefèvre, 2009; Huang *et al*., 2018). Additionally, it has a protein binding efficiency of 99 % and because of high lipophilic character, its bioavailability is significantly enhanced by food intake (Djimdé & Lefèvre., 2009). The effect of this combination is said to be effective against the erythrocytic stage of the parasite (Pingale & Mangaonkar., 2013). Lumefantrine was selected as the model drug in this study for the development of a high-performance liquid chromatography (HPLC) method that would subsequently be used to determine it in dry blood spots (DBS) filters collected from malaria patients. Artemether was excluded due to its extremely short half-life but was earmarked for future studies. The structure of lumefantrine is shown in Figure 1.2.



528.939g/mol

Figure 1.2. Chemical structure of lumefantrine.

1.3 Principles of High-Performance Liquid Chromatography (HPLC)

HPLC is a widely used separation technique. Its wide spread use, is due to its suitability for separating semi/non-volatile compounds, high sensitivity and most importantly, its applicability in industry and scientific fields in the analyses of significant compounds such as proteins, carbohydrates and drugs (Skoog *et al*., 2007). HPLC achieves separation based on the relative distribution of the solutes between two phases i.e. the stationary and the mobile phases. The analytes/ molecules in a liquid (mobile phase) are forced into the column (stationary phase) and eventually reach the detector. Due to interaction between analytes and the stationary phase and depending on polarities, separation is made possible. The separation is also influenced by the type of mobile phase. Thus, a few factors must be considered when selecting the mobile phase which includes the ability of the mobile phase to completely dissolve analytes as well as its polarity. In addition to the mobile and stationary phases, HPLC systems also consist of the pump, the injector, the detector and a data acquisition system such as a computer (for chromatogram display and recording) as shown in the Figure 1.3. It was deemed "beyond the scope" in this study to describe the operations of the majority of HPLC components except the detector that was used due to its influence on the results that were obtained.

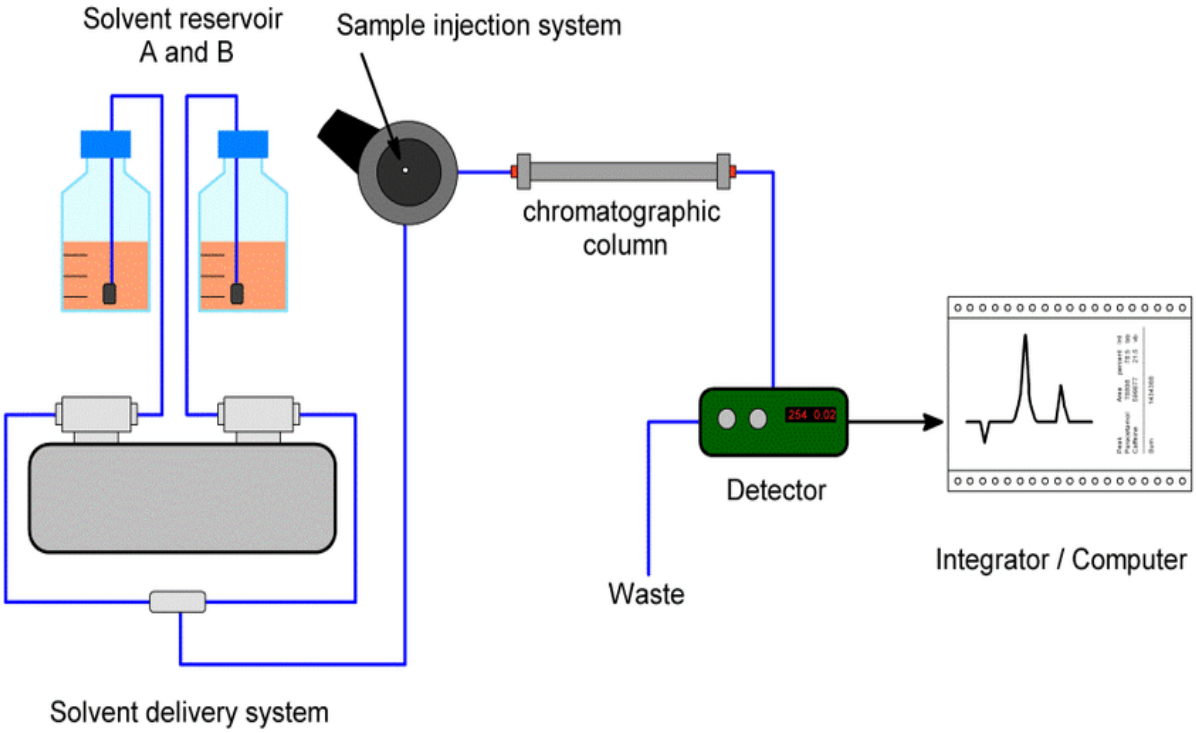


Figure 1.3. Schematic diagram of HPLC. Source:
https://www.researchgate.net/publication/307971644_Impurity_Profiling_of_Challenging_Active_Pharmaceutical_Ingredients_without_Chromophore

1.3.1 HPLC detectors

To date, HPLC separations have been done using a myriad of detectors. The most common ones are based on spectroscopic and spectrometric measurements. These include ultra-violet absorption, solid state, mass spectrometers and fluorescence detectors. The details of absorption detectors are given below to describe a specific diode array detector (DAD) which was used in this study. The DAD is solid state detector made of p-doped silicon superimposed on a a n-doped silicon substrate. The detector is encapsulated in a layer of silicon dioxide (SiO_2) which is insulating and upon which photons impinge as shown in Figure 1.4. On top of the p-doped silicon, are embedded an array of electrodes biased to a positive voltage say 10V for collection of electrons. When a photon strikes the n-doped silcone layer, and electron is released and since its negatively charged, it migrates to the positively charged electrode on the p-doped silicon side and

is collected below the elctrode. The hole after the electron is released is strored as a positive charge. This process repeats as more photons strike the n-doped silicon and as the elctrodes get saturated, a capacitor develops i.e. and charge is strores. This is the steady state of the detector. The electrons stored under each electrode are then read as a signal using the shift register as shown in Figure 1.4. A polychromator is used disperse a series of wavelengths each one focus on its own diode and as such many wavelenghts can be processed at one time. The DAD was used throughout this work following sample preparation which was accomplished by solid liquid extraction as described in Section 1.4.

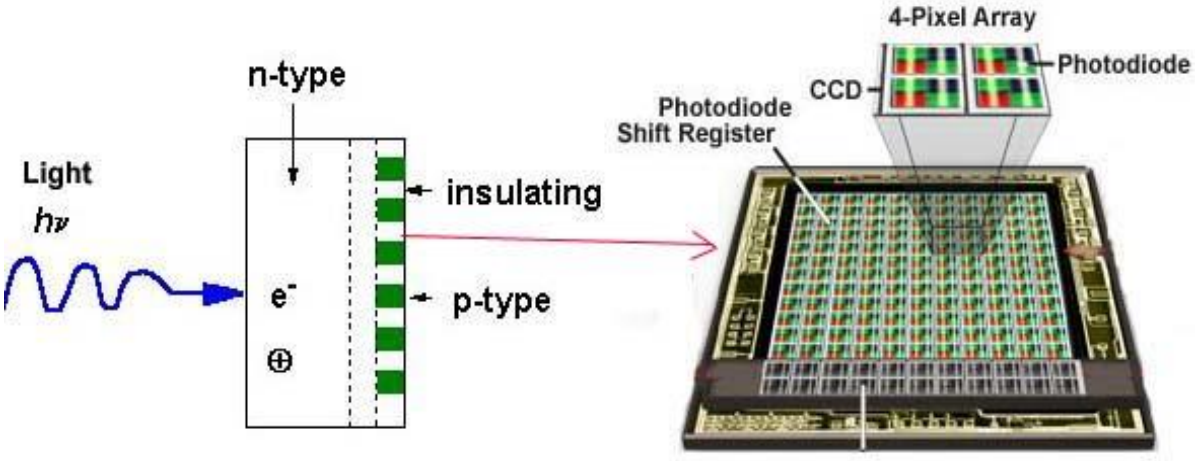


Figure 1.4. Schematic diagram of diode array detector. **Source:** http://www.globalsino.com/EM/page4910.html

1.4 Principle of Solid-Liquid Extraction (SLE)

SLE is based on the transfer of soluble solutes (analytes) from a solid matrix into a liquid through dissolution. The principles of this technique are like those guiding liquid-liquid extractions (LLE). The only different is that the matrix is a solid containing the solute/analyte of interest rather than a liquid. Just like in LLE, this procedure can also be optimized for critical factors in order to obtain good extraction efficiencies. These factors include the extracting solvent and the temperature at which the extractions are performed. The procedure utilizes a solvent with high

affinity for the solute molecules (analyte) than the undesirable impurities. The solvent should also be environmentally friendly, of low viscosity and have a low boiling point for ease of removal. This method was adopted and used in this study as described in Section 3.8.3.

2.0 LITERATURE REVIEW AND JUSTIFICATION

2.1 Analytical Methods for Determining Lumefantrine

Several analytical methods have been used and recommended. In all these methods the most significant stage that cannot be ignored is sample preparation. Sample preparation is attributed to 60-80% of work and operating cost and secondly it is estimated to be responsible for more than 50% errors associated with the final reported data (Ramos., 2012). From literature, the mostly widely used of these methods is HPLC hyphenated to different detectors as described below.

Silva and the group (Silva *et al*., 2015) analyzed lumefantrine (LUM) and Desbutyl-lumefantrine (DBL) in human plasma at Uganda using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and protein precipitate as the sample preparation method. The working ranges for LUM and DBL in this study were 21-529 ng/ml and 1.9-47 ng/ml respectively. The total chromatography run time was 2.2 minutes using a Hypersil Gold C18 column (20 x 2.1 mm, 1.9 µm) and a mobile phase consisting of water (A) and methanol (B) both with 0.5 % formic acid. 0.5 mL/mins was used as the flow rate. The method was applied in real samples of children under 5 years old with uncomplicated malaria up to 28 days after 3-day treatment with coartem (AL) to adduce pharmacokinetic data.

In Kampala, Uganda, Ntale *et al*., 2008 developed an HPLC method using UV detector to quantify lumefantrine and its metabolite, Desbutyl-lumefantrine in whole blood spotted on filter paper. Uganda is one of the African countries affected by malaria as well as having resistance by parasites towards the antimalarial drug. The chromatographic separation was performed using Zorbax Eclipse XDB-Phenyl column (4.6 mm x 150 mm with particle size of 5 µm) at a flow rate of 1.0 ml/min. Acetonitrile and ammonium acetate buffer (0.1 M ammonium acetate and 0.01 M

acetic acid. pH 6.5) were used as mobile phase at a ratio of 10:90 respectively. Absorbance's of the analytes were monitored at 335 nm wavelength. The extraction recoveries of the two analytes from the filter paper were averaged between 45-50 % and 25-33 % for LUM and DBL respectively, with the 300 nM as the limit of quantitation (LOQ) for both analytes. The method was applied in four patients treated with recommended dose of coartem (AL), they were aged 12, 12, 13 and 43 years old.

In one of the affected African countries, in which the malaria transmission is said to be stable and cases to have increased recently (World Health Organization., 2017), Tanzania at Tanga village, Khalil *et al*., 2011 developed an HPLC method for simultaneous measurement of LUM and DBL in human plasma using UV detector. The sample was extracted using liquid-liquid extraction (LLE) and separated using Synergi Polar-Reverse Phase (RP) column (250 mm x 300 mm, 4 µm particle size). The mobile phase consisted of acetonitrile - 0.1 M ammonium acetate buffer (pH 4.9), with isocratic elution of 85:15 respectively pumped at a flow rate of 1 ml/min. A wavelength of 335 nm with a reference of 360 nm was used to monitor the absorbance of the compounds. Extraction recoveries were 88% and 90% with LOQ of 12.5 and 6.5 ng/ml for LUM and DBL respectively. After the validation the method was applied in samples of patients with uncomplicated malaria and under treatment with coartem.

In Pakistan, a RP- HPLC method was developed and validated for simultaneous determination of LUM and DBL in human plasma by Khuda *et al*., 2014. They used Supercol Discovery HS C18 RP (150 mm x 4.6 mm, 5 µm) column to achieve the resolution of the compounds. They also used isocratic elution of 70:30 consisting of acetonitrile and 0.05 %trifluoroacetic acid respectively as the mobile phase, extracting the compound using Solid Phase Extraction (SPE). The mobile phase was pumped at a flow rate of 1.0 ml/min and absorbance

monitored at a wavelength of 335 nm. They report their method to show linearity over concentration range of 10 – 12,000 ng/ml. Their method gave a limit of detection (LOD) and lower limit of quantification (LLOQ) for LUM and DBL respectively as follows; 10.0 and 18.0 ng/ml and 7.5 and 15.0 ng/ml. This developed and validated method was applied for pharmacokinetic studies.

In the same vein, LC-MS/MS for simultaneous quantification of artemether and lumefantrine was developed and validated by César and the team (César et al., 2011). Their method consisted of protein precipitate as their extraction technique and reverse phase Zorbax SB-Ciano column with a binary mobile phase composed of methanol and 10 mM ammonium acetate (spiked with 0.2 % and 0.1 % v/v of acetic and formic acid) for separation. The mobile phase was delivered at a flow rate of 1.0 mL/min using gradient elution conditions. The resulting lower limit of quantification for both drugs was 10 ng/mL. They also applied the method in studying the pharmacokinetics in healthy volunteer's plasma.

In another study (Huang *et al*., (2010), an HPLC-UV method was developed and validated for the determination of lumefantrine in plasma. The extraction of compounds was carried out starting with protein precipitation followed by solid phase extraction in a sequence. The separation of compounds was performed with Zorbax SB-CN column (3.0 x 150 mm x 3.5 µm) and water: methanol (0.1 % trifluorocetic acid) as the mobile phase distributed at gradient elution conditions. Both the internal standard (halofantrine) and lumefantrine were monitored at 335 nm, and the linearity was between ranges of 50-10,000 ng/mL. The method was validated for its precisions and accuracy which were said to be within the acceptable range according to the AIDS Clinical Trials Group (ACTG) guidelines. Their method had mean recovery ranging between 84.0-88.2 % for all the analytes and was successfully applied in a pharmacokinetic study.

Motivated by the aim to apply the method in bioequivalence study; Pingale and Mangaonkar, developed and validated a LC-MS/MS method for quantification of lumefantrine (Pingale & Mangaonkar., 2013). They used protein precipitation for extraction of lumefantrine and artesunate (as internal standard) from human plasma and used inertsil ODS column for their chromatographic separation. Their method involved use of isocratic elution condition with detection performed in positive ionization mode. Validation was done with respect to precision, linearity and recovery. With a total analysis of 2.5 mins, their method linearity ranged between of 200-20,000 ng/mL with recoveries of 93.16 % and 91.05 % for LUM and artesunate respectively.

A LC-UV method was developed and validated in relation to determining lumefantrine and its metabolite. Conducted by Lindegardh and the group (Lindegårdh *et al*., 2005), they used automated solid phase extraction to recover the analytes from the plasma. They used SB-CN (250 mm x 4.6 mm) column with a binary solvent system consisting of a of acetonitrile-sodium phosphate buffer (pH 2.0; 0.1 M) (55:45, v/v) and a 0.05 M sodium perchlorate. This mobile phase was pumped at a 1.2ml/min rate with a detection monitored at 335 nm. Their limit of quantification was 0.024 and 0.021 µg/mL for lumefantrine and Desbutyl-lumefantrine respectively, unfortunately the method was not applied in pharmacokinetic study.

Suffice to mention that the literature on the determination of lumefantrine in human samples is very scanty as seen in Table 2.1.

Table 2.1. Different HPLC methods used to quantify lumefantrine, its metabolite and or artemether

Reference	Method	Matrix	Sample Extraction	Run time (mins)	Analytes	LLOQ (ng/ml)	Country	Application
(Silva *et al*., 2015)	LCMS/MS	H. Plasma	PPT	2.2	LUM & DBL	21 LUM, 1.9 DBL	Uganda	LUM and DBL quantification in Human plasma of children under 5 years old with uncomplicated malaria in a drug pharmacokinetic study.
(Ntale *et al*., 2008)	HPLC-UV	Dry Blood Spot	LLE	10	LUM & DBL	Both at 158.7	Uganda	Pharmacokinetic study of LUM and DBL in Plasma of four patients aged 12, 12, 13 and 43 years old.
(Khalil *et al*., 2011)	HPLC-UV	H. Plasma	LLE	About 10	LUM & DBL	12.5LUM, 6.5 DBL	Tanzania	Efficacy and Pharmacokinetic interactions of AL and antiretroviral drugs in adults (HIV/AIDS patients) treated for uncomplicated malaria.

(Khuda *et al.*, 2014)	RP-HPLC-UV	H. Plasma	LLE	10	LUM & DBL	18.0 Lum, 15.0 DBL	Pakistan	Determination of pharmacokinetics of LUM in healthy volunteers.
(César *et al.*, 2011)	LC-MS/MS	H. Plasma	PPT	About 8	LUM and ART	10 both	Brazil	Applied for determination of plasma concentration of the two drugs in healthy volunteers receiving a fixed dose combination.
(Huang *et al.*, (2010),	HPLC-UV	H.Plasma	PPT and SPE	20	LUM	Not defined statistically	San Francisco	Method was applied for pharmacokinetic study, of 13 healthy volunteers receiving 6 doses of coartem.

(Pingale & Mangaonkar., 2013)	LC-MS/MS	H. Plasma	PPT	2.5	LUM	200	Mumbai	Applied in sixty healthy volunteers under fasting condition and administered with single dose of 120 mg lumefantrine.
(Lindegårdh *et al*., 2005)	LC-UV	Plasma	Automated SPE	20	LUM & DBL	24 LUM, 21 DBL	Not necessary	Not applied in pharmacokinetic study

H: human; PPT: Protein Precipitate; LLE: Liquid-Liquid Extraction; SPE: Solid Phase Extraction; AL: Artemether-Lumefantrine

It is evident from Table 2.1, that HPLC is the most common separation method. This is due to its appropriateness for analysis of thermally labile compounds mostly pharmaceuticals and its flexibility in terms of optimization of the separation procedure. Literature review showed one report on the use of Gas Chromatography (GC) to determine lumefantrine, through derivatization. This study was conducted by Suleman and co-workers (Suleman *et al*., 2015) focusing on determining lumefantrine in pharmaceutical products. They obtained a limit of detection of 0.01µg/mL, which was lower than those obtained by HPLC tabulated in Table 2.1. Despite the low detection limit reported in that study, derivatization can have many shortcomings especially as far as selection of derivatization reagents is concerned. Some reagents are highly reactive and can inactivate the column leading to frequent column trimming to maintain chromatographic consistency. GC is not also suitable for of thermally labile drugs.

Also, evidently from Table 2.1, the application of LC-MS/MS and LC-UV are central in the analysis of lumefantrine, and both methods are widely applied to more or less the same extent. LC-MS/MS identifies the compounds by mass to charge ratio (*m/z*) of both precursor and product ions as well as the compound retention times (t_r) thus affording a two-dimensional data analysis function than LC-UV which only utilizes retention time as the only identification parameter. This does not mean LC-UV is unusable in such analysis, because it can still produce data that is comparable to LC-MS/MS depending on the study design. Despite it being less sensitive than LC-MS/MS which can detect up to trace levels, LC-UV is one of the traditional quantification and identification method that it is cheap and requires less maintenance compared to LC-MS/MS.

There is scanty literature on biological matrices such as DBS, as seen in Table 2.1 where only Ntale and the Co-workers (Ntale *et al*., 2008) determined lumefantrine in DBS. DBS is a biotechnology invention in which whole blood drops are spotted on to a filter paper. This invention and application of collecting blood on filter papers dates back to the early 1960s and has

been adopted recently. This growth emanates from its simplicity as a means of sample collection, storage and transportation over conventional whole blood, plasma and serum (Li and Tse., 2010; McDade., 2014; McDade *et al*., 2007; Wilhelm *et al*., 2014). Its collection is said to be minimally invasive, relatively painless with low risk thus simplifies subject's recruitment (Li and Tse., 2010; McDade., 2014; Parker *et al*., 1999) in studies like this one. Unlike other biological matrices, DBS filters do not need laborious processing such as separation, freezing and centrifugation (Li and Tse., 2010; McDade., 2014). Thus, DBS filters afford several advantages in the sample preparation stage which can present several hurdles in analytical method development. Unlike other biological matrices that are liquids (whole blood, plasma etc.), DBS filters do not require sophisticated conditions for storage and shipping, since they can be sealed into envelopes under room temperature and transported, thus reducing costs (McDade., 2014; McDade *et al*., 2007). Despite few advantages presented by DBS filters, some analytes may be altered due to drying as well as failure to measure some analytes due to presence of interfering components such as red and white blood cells (McDade., 2014; McDade *et al*., 2007). In this study, the simplicity of DBS filters and the advantages herein described, motivated their use in combination with LC-UV. This would greatly reduce the burden of sample preparation especially for method development and validation. Thus, the aim of this project was to utilize whole blood samples collected on BDS filters in a simple solid liquid extraction procedure to afford extracts that would enable the determination of lumefantrine using LC-UV. The method developed would then be applied to real DBS filter samples collected from malaria patients in Botswana. From literature review, this is the first project in Botswana to adduce DBS lumefantrine concentrations using a developed and validated LC- UV method.

2.2 Problem statement

Botswana adopted the use of ACTs, particularly coartem (AL) drug in 2007 as a first line treatment changing from chloroquine (CQ) and sulphamethoxsazol-Pyremethamin (SP). This change occurred after the efficacy test failure of these two drugs around mid-1990s. Botswana continues to experience malaria cases and deaths, even with the current ACTs treatment. The country is also facing the problem of resistance by the parasite towards ACTs which are said to be the best and effective as far as malaria treatment is concerned. According to (Bloland., 2001) the mismatched pharmacokinetics in clinical studies can facilitate the development of this problem of resistance. The problem could partly be due to the lack of pharmacokinetic data in this country. This project was designed to create a database of analytical methods capable of producing concentration results for lumefantrine in human samples which could be used for therapeutic drug monitoring (TDM) and pharmacokinetic profile studies of lumefantrine in Botswana.

2.3 Objectives

2.3.1 Main Objective

To develop, validate and apply a bio analytical method to determine the concentration of lumefantrine in dry blood spots (DBS) samples collected from selected locations in Botswana.

2.3.2 Specific Objectives

- To develop a LC-DAD procedure for the determination of lumefantrine using analytical standard.
- To adopt and modify extraction procedure for Dry Blood Spot (DBS) filters.
- To validate the method for quantification of lumefantrine in DBS filters.
- To apply the method in real samples collected from selected districts of Botswana.

CHAPTER TWO

3.0 EXPERIMENTAL

3.1 Team Composition and Safety Training for Human Sample Handling, Storage and Disposal

This project was done in collaboration with staff from the University of Botswana-University of Pennsylvania (UB-UPenn) Partnership. The collaboration between the Department of Chemistry (UB), the School of Medicine (UB) and UPenn staff, consisted of professionals as well as experts relevant to this study. Safety training on human sample handling, storage and disposal was undertaken due to the biological matrix involved i.e. Human dried blood spot (DBS) filters. The theoretical and practical training were offered by School of Medicine at UB and the Botswana Harvard Institute Partnership for HIV Research respectively. Vaccination against Hepatitis B (a three-dose schedule)" was administered to the students involved. The vaccination was done for a minimum of three dose schedule as prescribed by medical practitioners.

3.2 Sample Collection

DBS samples were collected on filters by medical/biomedical professionals and their criterion for inclusion was that all patients diagnosed with malaria using rapid diagnostic test (RDT) qualified for the study. All the blood spots were acquired through the University of Botswana-University of Pennsylvania (UB-UPenn) Partnership under the Botswana malaria program. All the samples were collected in accordance with the WHO guidelines under the malaria program. The guidelines (full set not provided to this study) included recording the baseline characteristics of the patients from which the samples were collected longitudinally from day 0, 3, 14 and 28. Twelve (12) DBSs samples were collected from two districts i.e. Central and Ngamiland districts. Nine (9) samples were from Ngamiland and three (3) were from Central district.

3.3 Sample Storage

The DBS filters were stored at room temperature and were punched into a centrifuge tube for extraction at the time of preparation.

3.4 Reagents and Materials

Lumefantrine (Lum) standard was purchased from United States pharmacopeia convention (Rockville, USA). Caffeine (Caf) used as the internal standards (IS) were supplied by Sigma Aldrich, St Louis USA. All solvents used were of analytical grade from different manufacturers. Acetonitrile used was supplied by Fisher scientific, Loughborough, UK and the formic acid (FA) of purity of 85% was supplied by Sigma Aldrich, St. Louis USA. N-hexane and ethyl acetate were supplied by Chemcity, (Gaborone, Botswana) and Minema, (Johannesburg, SA) respectively.

3.5 Apparatus

High purity water, used as mobile phase was processed using Heal Force Super series NW ultra-pure water system supplied by Analytic Instruments Co. (Shanghai, China), and the samples were centrifuged using Thermo scientific SL 16R centrifuge from Thermo Fisher Scientific (Bremen, Germany), . The vortexing was performed using shaking incubator SI-300 purchased from Jeio tech (Seoul, Korea).

3.6 Stock solutions

A 1000 ppm cocktail of Lum and Caf were prepared as stock solution: with acetonitrile containing 0.15% formic acid. The stock solution was stored in a refrigerator and protected from light by storing in amber bottle. The working standards were prepared by serial dilutions of the stock solutions in acetonitrile prior to analysis. All working standard were stored at 4ºC.

3.7 Instrumentation

3.7.1 High Performance Liquid Chromatography-Diode Array Detector (HPLC-DAD)

The concentrations of the targeted analytes were determined using an Agilent 1100 HPLC system coupled to a Diode array detector (Agilent technologies, Palo Alto, CA USA). This system consists of quaternary pump (G1311A), vacuum degasser (G1379A), autosampler (G1313A), and thermostated column compartment (G1316A) and diode array detector. The quaternary pump comprises of four solvent reservoirs connected to vacuum degasser.

3.7.2 High Performance Liquid Chromatography (HPLC)

Analysis in all the chromatographic separation was carried out using an XTerra column of dimensions 50 mm length x 4.6 mm internal diameter x 5 µm Particle size. Acetonitrile and water (adjusted to pH of 2.3 using FA) was filtered and degassed ultrasonically for 15 minutes. The following procedure was applied in the analysis. The gradient elution program used was as follows: initially acidified water was kept at 18% and increased rapidly to 50 % up to 0.91 minutes and allowed to return to the initial condition of 18 % in 1.09 minutes for a total run time of 2.00 minutes. The mobile phase flow rate was 0.570 mL/min. Throughout, the injection volume was 10µL. The analyses were performed at room temperature because the analyte under study is thermally labile. The Data analysis was performed using Productivity Chemstation software.

3.8 Method Development

3.8.1 Separation optimization

Separation was optimized first by using neat standards injected into the HPLC-DAD system to establish the retention times of individual analytes (LUM and IS), then a cocktail of the standards was prepared and injected into the HPLC-DAD to confirm the resolution. As part of the separation optimization, the flow rate optimized using a Van Deemter plot shown in Chapter 3.

3.8.2 Calibration method

The internal standard method was used for calibration. The internal standard was spiked in blank samples containing analyte prior to extraction. These samples were used for method validation through internal standard calibration method.

3.8.3 Solid-Liquid extraction (SLE)

The extraction procedure for DBS was adopted from Khalil *et al* and slightly modified to suit the needs (Khalil *et al*., 2011). Control samples (screened specifically for this study) of DBSs were used for validation. The DBSs devoid of any lumefantrine drug, were first spiked with the analyte and the IS prior to extraction. This afforded calculation of percentage recoveries. After spiking, 3ml of n-hexane/ ethyl acetate (70:30%v/v) was added. The mixture was vortexed for 15 minutes vigorously and further centrifuged for 10 minutes at 4000 rpm. The resulting organic phase was transferred and evaporated to dryness under a stream of nitrogen gas at room temperature. The residue was reconstituted in 3 mL of the selected mobile phase i.e. acetonitrile: water (pH 2.3) (80:20% v/v respectively), and vortexed for about a minute. The extract was then injected into the HPLC system for analysis.

3.9 Method validation

The United State Food and Drugs Administration (U.S FDA) guidelines were used for validating the method. The following analytical performance parameters were investigated: instrument detection limits (IDMs), method detection limits (MDLs), percent matrix effect (% ME), linearity (R^2), the interday and intraday variations; extraction efficiency, accuracy (defined as % mean of recovery), precision (defined as % relative standard deviation). After establishing that the method was fit for purpose, it was then applied to quantify Lum in real DBS filters.

3.9.1 Instrument detection limits (IDLs)

IDL is defined as the minimum concentration of an analyte in the solvent in absence of a matrix that can be detected by an instrument. To determine the IDL, a calibration curve consisting of seven different concentration points was constructed. The concentrations from 1-8 ppm of the analyte were prepared, and Caffeine added as IS in each so that its concentration was 2 ppm. Four determinations per concentration were done, and the results of peak area ratio of analyte to IS at these seven levels were used to construct a calibration curve. The following were calculated based on the resulting calibration curve: limit of detection (LOD), lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ). Note that these values are related to the IDL because only neat standards were used to adduce them.

3.9.2 Method detection limits (MDLs)

Recommended by the U.S FDA guidelines, MDL is defined as a measure of the smallest amount of analyte concentration in the presence of a matrix that can be detected. They can also be defined as the matrix effects limits. Similarly, seven concentration used in IDM above were also adopted for determining MDLs. In addition to the concentrations used; 1- 8 ppm, a zero calibrator (a blank plus internal standard (IS)) was added as recommended by U.S FDA. At each concentration level

including a zero calibrator, a punch of control/ blank DBS filters was introduced. Vials were left for 30 minutes after which they were shaken vigorously for 2 minutes and injected. Four determinations were done for each concentration level and the results of peak area ratio of analyte to internal standard were used for the construction of calibration curves. Using the curves constructed, LODs, LLOQs and ULOQs were calculated using Equations 2.5, 2.6 and 2.7 respectively.

3.9.3 Percent matrix effect (% ME)

It is defined in U.S FDA guidelines as an alteration or interference in response by any unintended analytes present in the sample/ matrix (US Food and Drug Administration., 2018). This can also be defined as the percent mean deviation of the signal between IDLs and MDLs. This was determined using signals from both IDLs and MDLs, and was calculated using the equation 2.1 below adapted from Matuszewski and the group (Matuszewski *et al*., 2003).

$$(\%)\mathrm{ME} = \left(\frac{\mathrm{B}-\mathrm{A}}{\mathrm{A}}\right) \times 100\% \qquad (2.1)$$

Where B is the average peak area of the matrix standard and A is the average peak area of the solvent standard.

3.9.4 Selectivity

The U.S FDA guidelines (US Food and Drug Administration., 2018) define selectivity as the ability of the method to discriminate interferences from the analyte compound. This is done by analyzing blanks from at least six distinct individual samples and should be of the same matrix in which the analyte is contained. The absence of interference, such as ghost peaks especially at the retention times of both analyte and the internal standard qualifies the method to be referred to as selective.

3.9.5 Linearity

Linearity is measured using R squared (R^2) value. This value is known as the coefficient of determination; it indicates how well the current data fits a statistical model. This value ranges between 0 and 1, but the most desired values are those close to 1 for they assure the fitness of the data to the given statistical model. To test for this parameter a calibration curves for both IDLs and MDLs were used.

3.9.6 Extraction efficiency

Extraction efficiency estimated as percent recovery is a measure of the efficiency of an extraction technique. To determine the efficiency, blank DBS filters were spiked with known concentrations of the analytes at three concentrations levels estimated using the MDLi.e. at the LOD, LLOQ and ULOQ. These levels were estimated to be 0.4, 0.8 and 1.3 µg/mL respectively with internal standard (caffeine) spiked at a concentration of 5 µg/mL in all the three volumetric flasks. The extraction was carried out using SLE and three replicates for each level were made with four determinations per replicate. Recoveries were calculated using Equation 2.2.

$$\%\ \text{recovery} = \frac{\text{Recovered concentration}}{\text{Spiked concentration}} \times 100\% \qquad (2.2)$$

3.9.7 Accuracy

This involved spiking the blank DBS filters at three concentrations around or near the MDLs i.e. 0.4, 0.8 and 1.3 ppm as LOD, LLOQ and ULOQ respectively, followed by applying the SLE procedure. Three replicates per concentration and four determinations per replicate were made. The accuracy which sometimes is equated as % mean recovery was calculated as the percentage deviation of the mean value from the true value. This was calculated using Equation 2.3

$$\% \text{ Accuracy} = \frac{|\text{True value} - \text{mean value}|}{\text{True value}} \times 100\% \quad (2.3)$$

This was done for calculating both the intraday and interday accuracies as recommended by the U.S FDA guidelines (USFDA Guidelines, 2018). The USFDA guideline states that the deviation should not exceed $\pm$15% at the ULOQ except for LLOQ where they should not exceed $\pm$20% of the nominal concentration.

3.9.8 Precision

Precision is defined as the coefficient of variation (CV), commonly calculated as relative standard deviation RSD. It was calculated using Equation below.

$$\text{RSD} = \frac{100s}{\bar{X}} \quad (2.4)$$

Where s is the standard deviation and $\bar{X}$ is the mean value of all the data sets. U.S FDA guidelines recommends that precision i.e. % RSD should deviate by not exceed $\pm$15% CV at ULOQ level except at LLOQ where it should deviate by $\pm$20% CV.

3.9.9 Limit of detection (LOD)

LOD also known as minimum detectable value it was defined as the lowest concentration of the analyte that is distinguishable from the blank and can be detected at a specified confidence level. Statistically it was calculated using Equation 2.5 below.

$$\text{LOD} = \frac{3S_{XY}}{\text{m}} \quad (2.5)$$

Where S_{XY} is the STEYX function in excel. It is the standard error (SE) for the line of the best fit through a supplied set of x- and y- values i.e. the line of regression of y on x. m is the slope/ gradient of the line of regression under a given set of x and y values.

3.9.10 Limit of quantification (LOQ)

This is also known as determination or reporting limit. It is the "lowest concentration level of the analyte at which the measurement is quantitatively meaningful" (Saadati *et al*., 2013). With this limit, there is the lower limit of quantification (LLOQ) and the upper limit of quantification (ULOQ). Researchers have various ways of calculating the LLOQ and ULOQ but for this work the following equations were used.

$$\mathrm{LLOQ} = \frac{6S_{XY}}{\mathrm{m}} \qquad (2.6)$$

$$\mathrm{ULOQ} = \frac{10S_{XY}}{\mathrm{m}} \qquad (2.7)$$

CHAPTER THREE

4.0 RESULTS AND DISCUSSION

4.1 Method Development

The development of the LC-DAD method was divided into three phases. The first phase was the optimization of separation parameters. The second phase was adoption and modification of the sample preparation procedure and the third phase was validation of the method using the United States Food and Drug Administration (USFDA) guidelines. After the method was assembled, it was then applied to the quantification of Lum in DBS samples. In order to optimize the separation parameters, the following were considered:

(i) Selection of the wavelength of absorption

(ii) Selection of the analytical column

(iii) Selection of the mobile phase

(iv) Optimization of the flow rate.

Results for the optimization of separation parameters are discussed below.

4.2.1 Selection of the wavelength of absorption

Due to the principle of operation of the DAD, maximum absorption wavelength with respect to compounds investigated needs to be established for their identification and quantification. The selection of the maximum absorption wavelength (λ max) was done by scanning for the maximum wavelength of Lum and Caf using a double beam scanning UV/VIS spectrophotometer. Maximum wavelengths for both Lum and Caf were observed at 242 and 270 nm respectively dissolved in acetonitrile: water (with pH adjusted to 2.3).

4.2.2 Selection of the analytical column

Selection of the analytical column was limited by availability. Our laboratory had a stock of similar C18 XTerra columns manufactured by the Waters Corporation. From literature, this family of columns have never been used for the separation of Lum, so a decision was made to run preliminary experiments with them. Results of these preliminary experiments using the XTerra (50 mm x 4.6 mm x 5µm) column showed good resolution between Lum and Caf peaks with no interfering ghost peaks.

4.2.3 Selection of the mobile phase

Acetonitrile: water adjusted to pH 2.3 using formic acid (FA) gave the best peak resolution compared to methanol (result not shown) which was also tested. Figure 4.1 below shows a chromatogram that was obtained using acetonitrile: water as the mobile phase.

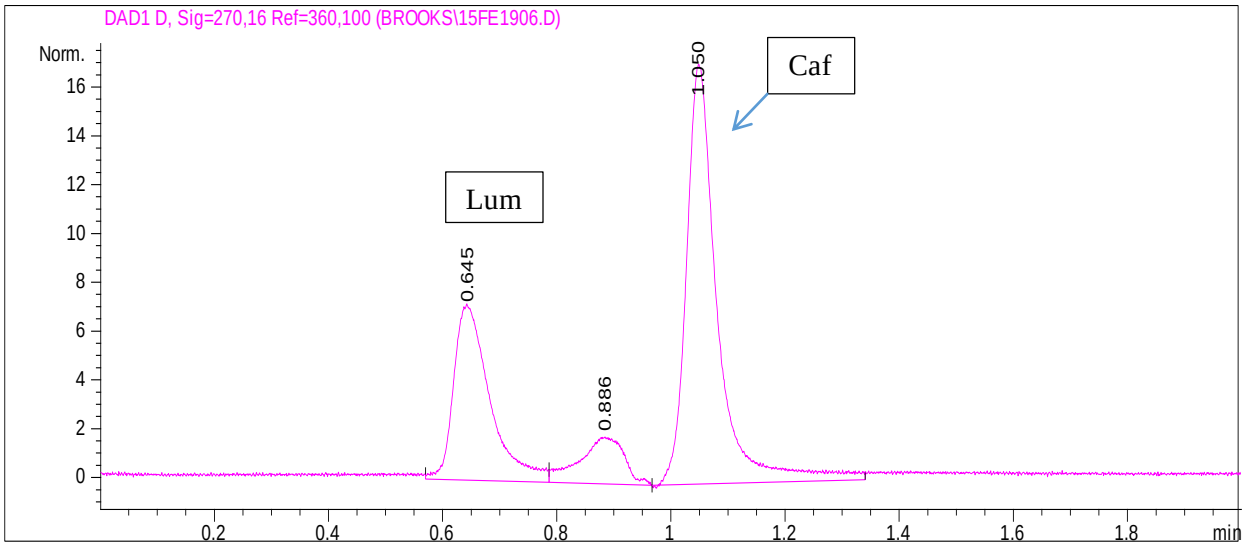


Figure 4.1 .Chromatogram of a mixture of Lum and Caf standards at 1 and 2 µg/mL respectively

The chromatogram in Figure 4.1set the pace to optimize the flow rate of the system in order to realise optimum separation and quantification values.

4.2.4 Optimization of the flow rate

This was done by investigating several gradients that were formulated based on the polarities of the analyte and the internal standard. The flow rates were varied one at a time using the gradient describe in Section 3.7.2 and both the number of theoretical plates and resolutions for Lum were recorded. This data was then converted to the relevant units for construction of the van Deemter plot for Lum only. Table 4.1 below shows the flow rate, the corresponding resolutions and number of theoretical plates (N) obtained while incrementing the flow rate in steps of 0.2 mL starting with 0.200 mL.

Table 4.1. Effect of flow rate on the number of theoretical plates (N) and resolution.

Flow rate (ml/min)	N	Resolution
0.200	638	0.87
0.400	658	0.95
0.600	695	0.96
0.800	492	0.83

Figure 4.2 shows the corresponding van Deemter plot for Lum which was plotted to confirm the optimum flow rate of the separation.

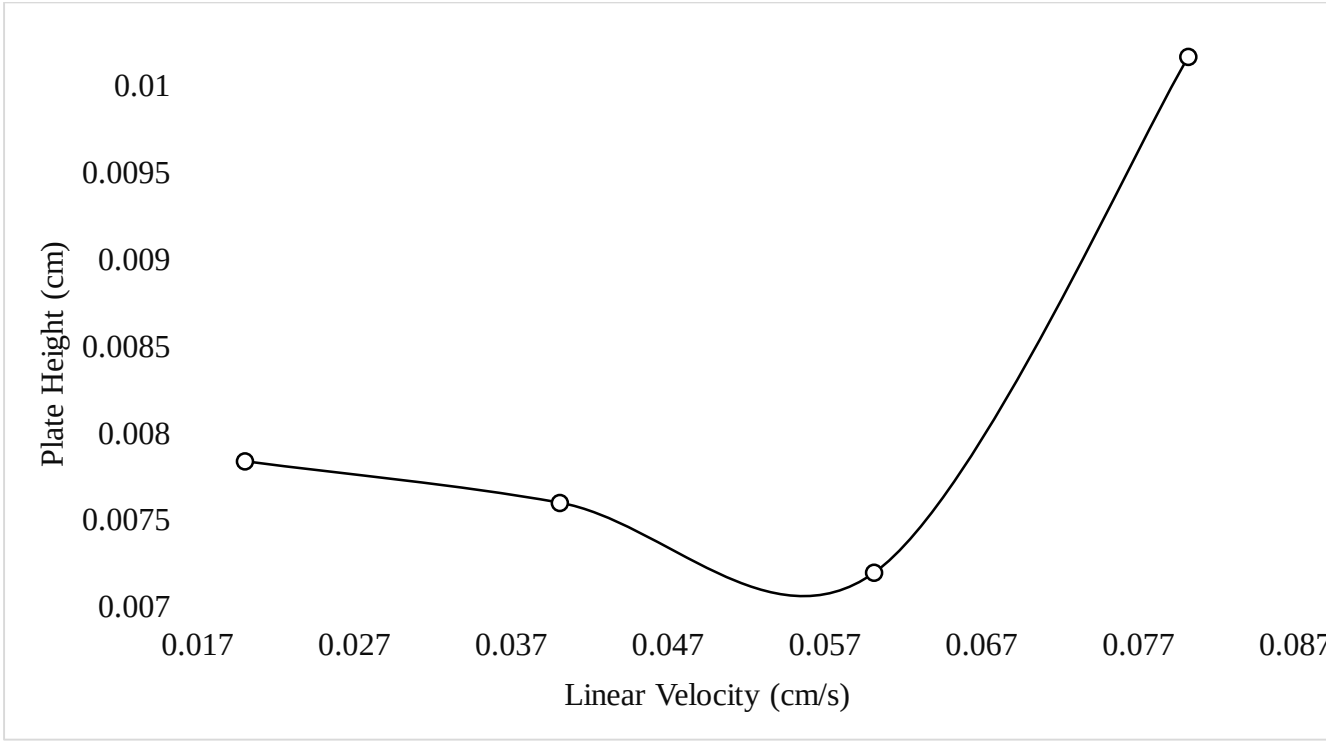


Figure 4.2.van Deemter plot for Lumefantrine.

From Figure 4.2, the linear flow velocity corresponding to the minimum plate height was taken as the optimum flow rate. Through extrapolation of points, this was located at a linear flow rate of 0.057 cm/s with the plate height of 0.00706. These two values are equivalent to a volumetric flow rate of 0.570 mL/min and number of theoretical plates of 708 respectively. These flow rates are indicative of optimum interaction between the analyte and the stationary phase which is influenced by the mobile phase solvent strength that allows for their separation.

4.2.5 Summary of Optimized Separation Parameters

After optimizing the parameters of interest including the separation gradient, a summary of the same is given in Table 4.2.

Table 4.2. Optimum gradient composition.

Time (mins)	Flow rate (mL/min)	% Acetonitrile	% Water (2.3 pH)
0.0	0.570	82	18
0.90	0.570	50	50
2.0	0.570	82	18

4.3 Adoption and Modification of the Sample Preparation Procedure

After implementing the SLE sample preparation procedure described in Section 3.8.3 on a spiked blank DBS filter and the optimized parameters in Table 4.2, the chromatogram shown in Figure 4.3 was obtained. Experiments under this study were all done using the acetonitrile and water (pH 2.3) mobile phase system.

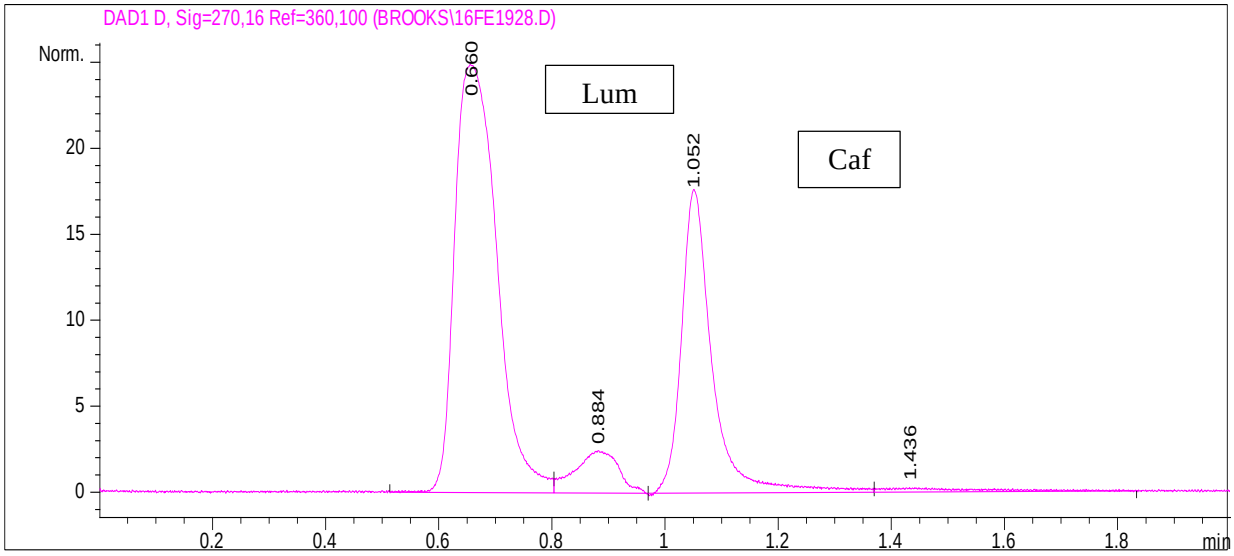


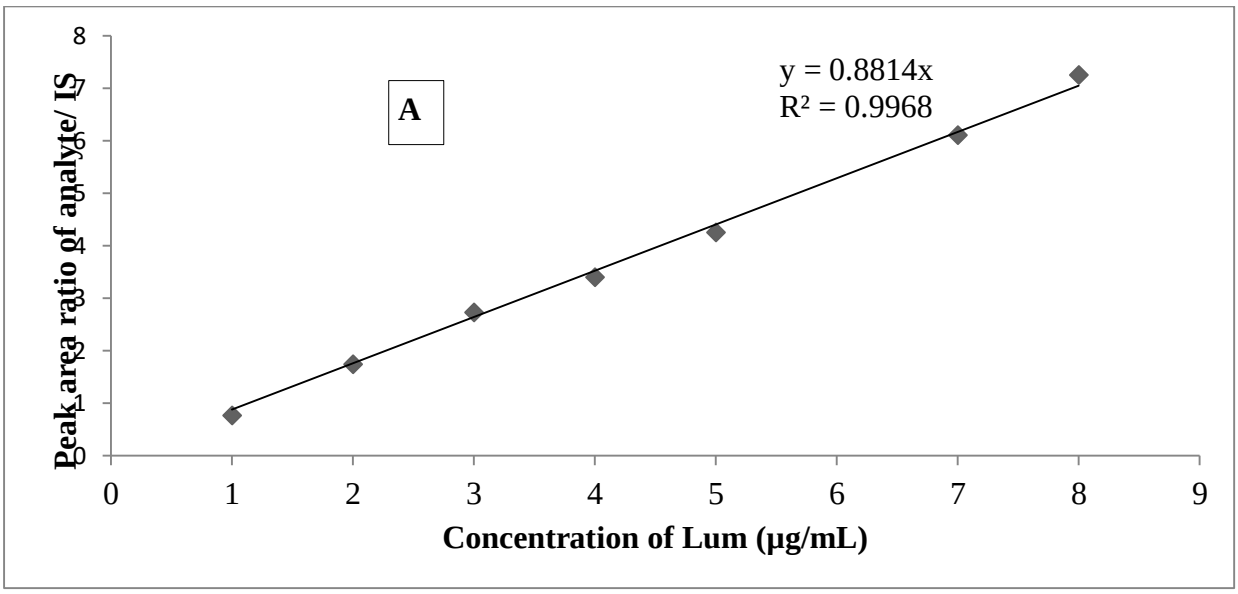
Figure 4.3. Chromatogram of a DBS extract spiked with a cocktail of Lum and Caf at 4 µg/mL and 2 µg/mL respectively

4.4 Method Validation

Method validation was done using the United States Food and Drug Administration (USFDA) guidelines (USFDA, 2018). In a nutshell, the USFDA guidelines describe bioanalytical method validation as a process that proves that the optimized method is suited to the analysis of the study samples. The guidelines further enlist some but not all the parameters that require optimization as the calibration curve, quality control samples (QCs), the selectivity and specificity, sensitivity, accuracy, precision and recovery. In this study, the instrument detection limits (IDLs) and the method detection limits (MDLs) for the determination of Lum were estimated as part of method validation. Other parameters that were estimated were the selectivity, the specificity, the sensitivity, the accuracy and the precision of the method. The recoveries were also adduced which in a nutshell are related to the accuracies. The results from method validation will be discussed in the sub-sections that ensue.

4.4.1 Calibration curves

Calibration curves were constructed using seven points. The curves afforded the estimation of IDLs which were used as footprints for spiking blank DBS filters to enable calculation of recoveries, the accuracy and the precisions. Later, MDLs were also estimated based on the spiked blank DBS extracts. Figure 3.4 shows calibration curves using pure or neat standards and a calibration curve prepared in the matrix of interest as prescribed by the USFDA guidelines. The calibration curve in (b) i.e. with the matrix was used throughout the rest of the experiments.



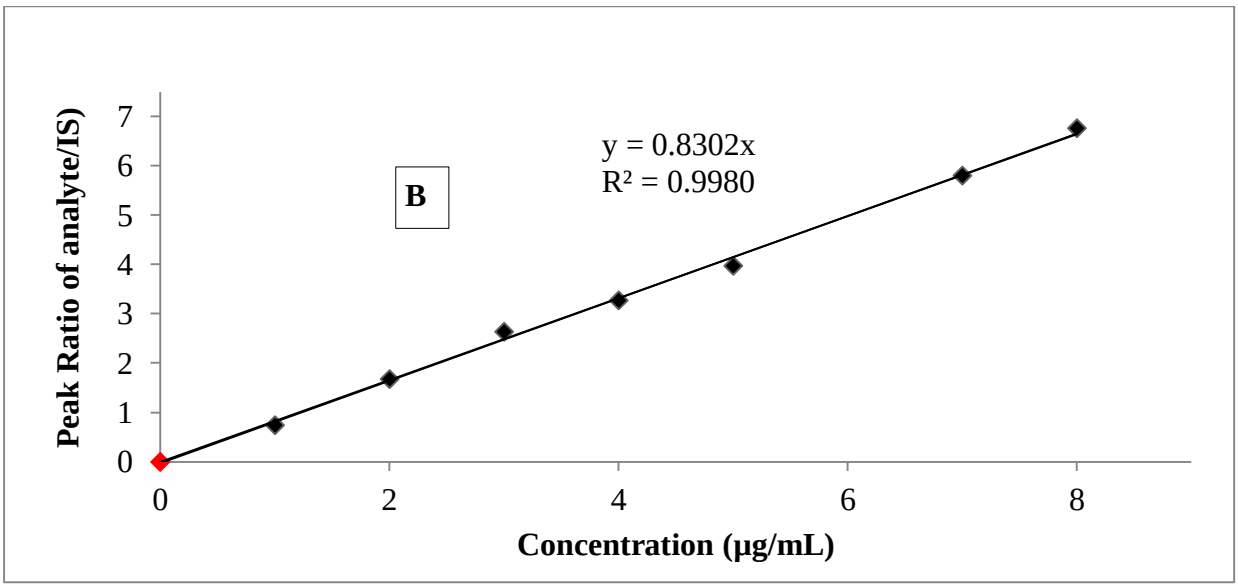


Figure 4.4. Both calibration curves of lumefantrine i.e. (a) neat standards and (b) matrix under study.

4.4.2 Linearity

Both calibration curves i.e. with neat standards and with matrix were linear with coefficients of determination of 0.9968 and 0.9980 respectively as shown in Figure 4.4. These were taken as very good linearities.

4.4.3 Instrument detection limits (IDLs)

The IDLs provide information about the responsivety of the instrument which is an indication of instrument performance at the time the instrument is used. IDLs were estimated using the Equation 2.5.

Table 4.3. Instrument detection limits (IDLs).

	Regression Equation	**R^2**	**IDL (ppm)**	**LLOQ (ppm)**	**ULOQ (ppm)**
Lumefantrine	$y = 8814x$	0.9968	0.4	0.9	1.5

The IDLs were reasonably low suggesting that the instrument was in optimal condition to detect the analytes. This was attributed to the absence of matrix interferences in the neat standards, which commonly reduces these IDLs. These values were determined from the calibration curve given in Figure 4.4 (a) i.e. in which neat standards were used as opposed to the one that contained the matrix of interest. Thus, in this case, there was noise interference emanating from the instrument only rather than the matrix.

4.4.4 Method detection limit

The USFDA recommends that the calibration curve at a working range should be constructed with analyte in a matrix to establish the effects of the matrix. The MDLs were derived from the calibration curve constructed using the matrix and provided real detection limits or limits of detection (LODs) of the method i.e. from the matrix of interest present in the blank sample as provided for in the USFDA guidelines. Thus, the values account for both noises from the instrument and the matrix. The MDL thus establishes the impact of a matrix on the instrument signal/ response. Seven concentrations as well as a zero calibrator were prepared in the presence of a matrix of interest in order to evaluate these limits as shown in Figure 4.4 (b). The results are given in Table 4.4.

Table 4.4. Method detection limits (MDLs) equivalent to limit of detection (LODs).

	Regression equation	**R^2**	**LOD (ppm)**	**LLOQ (ppm)**	**ULOQ (ppm)**
Lumefantrine	$y = 8302x$	0.9980	0.4	0.8	1.3

Generally, in comparing the limits, the deviation observed between them suggest that the extent to which the matrix impacted the response was not significant. The R^2 values for both calibration curves where the IDL and MDL were estimated, were greater than 0.9900 suggesting good proportionality and linearity between concentration and the signal signifying minimal or no matrix interference. As seen from Table 4.4 and 4.5, the IDL was the same as the MDL (in this case was equivalent to the LOD). The small difference between the limits of detection was speculated to be due to absence of interferences that might have suppressed the signal to a large extent, and also the introduction of minute amounts of matrix in the HPLC system did not introduce significant matrix interferences. This is a figure of merit for using DBS filters to collect human blood samples.

4.4.5 Percent matrix effect

This parameter indicates the extent to which there is a direct or indirect interference of the response by an interfering substance(s) (U.S FDA, 2018). The extent to which interferences impact the response either positively or negatively is not specified in the USFDA guidelines, except the selectivity which is also an indication of matrix interferences. The guideline under selectivity, recommends that the response in both the blank matrix and calibrators should not deviate by more than 5%. This was adopted to work out the acceptance of the percent deviation for matrix effect. Percent of matrix effects are tabulated in Table 3.5. These values were estimated using Equation 2.1.

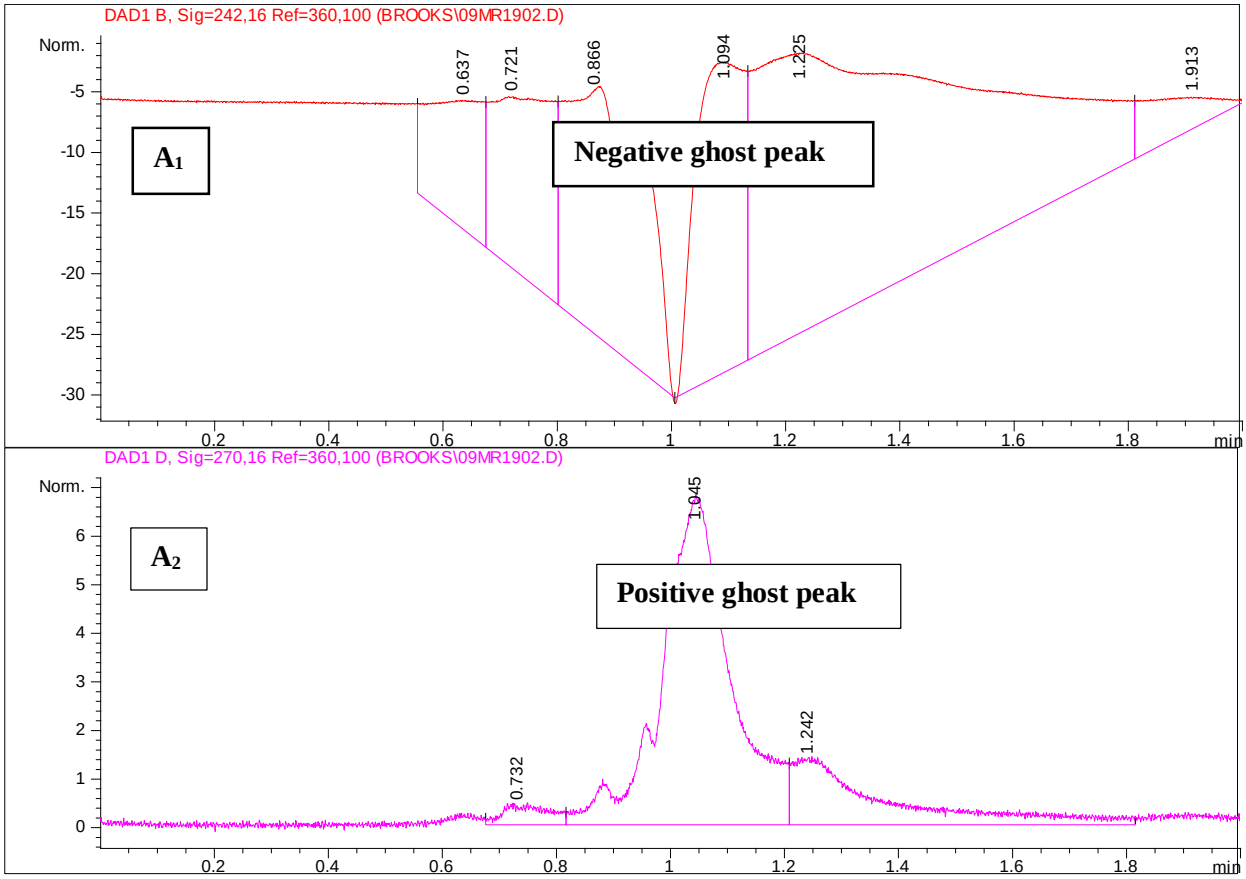
Table 4.5. Percent matrix effect

Concentration (ppm)	% Matrix effect
1	-4.7
2	-4.7
3	-3.6
4	-3.8
5	-6.7
7	-5.1
8	-6.9

Using the recommendation for selectivity from the guidelines, from Table 3.5, all the deviations were negative which indicated that there was suppression of the response within the range except the last three which deviated by more than the 5%. This indicated signal suppression/reduction which is common in bio analysis especially using instruments such as HPLC- DAD systems which are not as specific.

4.4.6 Selectivity

Selectivity, as recommended by the U.S FDA guidelines, was investigated by analyzing blanks (i.e. no internal standard and lumefantrine) of dried blood spot filters from six distinct individuals. This was investigated at two wavelengths 242 and 270 nm corresponding to Lum and Caf respectively. Figure 4.5 shows two chromatograms from two DBS filters of different individuals. Other chromatograms are included in the appendices.



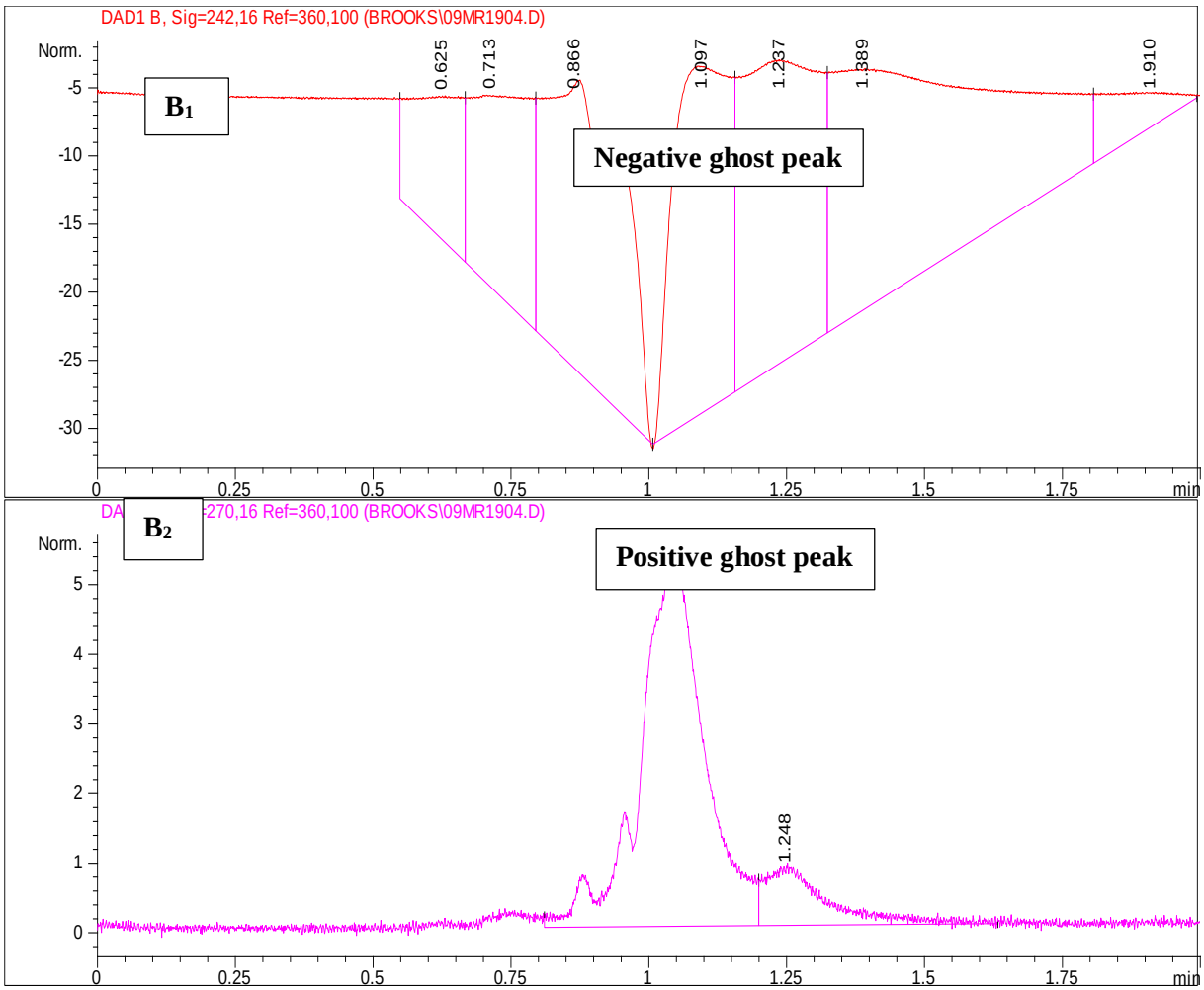


Figure 4.5. Chromatograms (A1, A2 and B_1, B_2) are for the blank DBS samples from two patients used to study the selectivity of the method at two wavelengths;(red chromatograms monitored at (242 nm) and pink chromatograms monitored at (270 nm).

It was noted from these chromatograms that both negative and positive ghost peaks were observed within the analysis time. Negative peaks were ascribed to the variation of the mobile phase composition throughout the analysis. The other speculated causative effect was due to optical effects. These peaks were suspected to be low concentrations of caffeine that was present in the blanks. Confirmation for this observation was made using a pure/neat standard chromatogram of caffeine which was only observed at 270 nm. This suggested that the subjects under the study had consumed food containing low amounts of caffeine. To resolve the caffeine interference, peak

area of this ghost peak, which showed a very minute effect/ had no significant effect (very low concentration/) were subtracted from the total peak areas.

4.4.7 Extraction efficiency expressed as percent recoveries

The extraction efficiencies also referred to as percent recoveries were also evaluated. These were estimated at three spike levels i.e. the LOD, LLOQ and ULOQ given in Table 4.4. The study involved spiking, extracting and analysis of the extracts at these three levels. Three replicates (n=3) for each level were made with four determinations per replicate. The recovery studies were performed in three days with an interval of three days apart. Equation 2.2 in Chapter two was used to calculate the recovery and results are tabulated Table 3.8

Table 4.6. Percent (%) recoveries at three spiked levels i.e. LOD, LLOQ and ULOQ.

LEVEL	DAY 1	DAY 2	DAY 3
LOD	61.7-73.1	69.7-78.7	74.3-77.6
LLOQ	79.6-89.1	85.3-87.4	85.6-89.2
ULOQ	101.2-123.9	95.0-127.5	94.2-109.2

All the recoveries were above 60% as seen in Table 4.6, which was reasonably good extraction efficiency for the extraction procedure. This was attributed to the simplicity of the extraction procedure that did not incorporate a clean-up step. This step usually lowers the recoveries due losses that ensure during the process. It was also observed that as the level of spiking increased, the recoveries also increased. It was speculated that at lower spike levels, the quantities of the analytes gave signals that were close to noise signals and differentiating the signals at those levels was difficult.

4.4.8 Precision expressed as percent relative standard deviation (% RSD)

As part of the validation strategy, precision was examined. It is commonly defined as the closeness of the experimental values to one another and sometimes referred to as the coefficients of variation (CV). It is calculated as the percent relative standard deviation (%RSD) using Equation 2.4 in Chapter two. The acceptance criteria as recommended by the guidelines for precision are similar as for accuracy in the UDFDA guidelines, thus the CVs at any spike level should not deviate by more than ±15% except at LLOQ where it should not deviate by more than ±20%. The within day reproducibility's are shown in Table 4.7.

Table 4.7. Within day precision expressed as % RSD.

	Day 1			**Day 2**			**Day 3**		
	LOD	LLOQ	ULOQ	LOD	LLOQ	ULOQ	LOD	LLOQ	ULOQ
CV	8.4	6.5	10.5	6.6	1.2	15.4	2.8	2.1	8.8
SD	5.7	5.4	11.6	5.0	1.0	16.8	1.8	1.9	8.8

SD= Standard deviation

The between days reproducibilities are shown in Table 4.8, and they were estimated from percent recovery values

Table 4.8. Between day precision estimated using percent recoveries and expressed as % RSD

LOO	3.3-11.4
LLOQ	2.3-5.6
ULOQ	4.0-7.9

All precisions were within the recommended limit of ±15% at the LOD and ULOQ spike levels and ±20% at the LLOQ spike level. This denoted good precision which was an indication of good reproducibility.

4.4.9 Accuracy

U.S FDA defines accuracy as the deviation of the determined value from the nominal value under given conditions (U.S FDA, 2018). The guidelines recommend that the accuracy at each level of spiking should not deviate by more than ±15% from the nominal concentrations except at LLOQ where they should not deviate by more than ±20%. These studies were carried out within and between days and the results are shown in the Table 3.6.

Table 4.9. Percent (%) accuracies.

LEVEL	DAY 1	DAY 2	DAY 3
LOD	26.9-38.3	21.3-30.3	22.4-25.7
LLOQ	10.9-20.4	12.6-14.7	10.8-14.4
ULOQ	1.2-24.0	4.1-27.5	5.3-9.6

Poor accuracies were obtained at the LOD spike level with all values exceeding the recommended level of less than ±15% as seen in Table 4.9. This was attributed to the difficulty to reproduce results at those low spike levels where extraction could either yield a value greater than or less than the LOD value. At the LLOQ, the values met the USFDA guidelines i.e. all values were within the ±20 % requirement. At the ULOQ, the accuracy spread was much wider than at the LLOQ and in some instances exceeded the ±15% requirement. Generally, these values suggested good accuracies despite some deviations which were attributed to minor systematic errors.

4.5 Application to the Analysis of Real DBS Samples

A series of tables, showing concentration averages of lumefantrine for 12 subjects (malaria patients) over a period of 28 days collected at different days are given below. Table 4.10 and 4.11 showing concentrations of patient from Ngamiland and central district respectively. The concentrations of lumefantrine were adduced using calibration graphs presented in 4.3.1.

Table 4.10. Average concentrations of Lum in DBS filters collected from Ngamiland

	DAY 0	**DAY 3**	**DAY 14**	**DAY 28**
Subjects Code	**Average Concentration (mg/L), (n=3)**	**Average Concentration (mg/L), (n=3)**	**Average Concentration (mg/L), (n=3)**	**Average Concentration (mg/L), (n=3)**
129	1284.03	1698.18	964.53	710.09
375	1339.19	1353.85	1140.35	834.48
376	2168.98	838.80	2997.51	1887.31
400	1554.40	625.16	510.33	650.44
576	1459.32	970.95	561.82	477.49
587	1101.90	1213.89	767.08	736.65
588	836.78	889.86	706.62	551.80
590	825.33	851.77	763.26	756.78
591	1108.27	1129.34	1073.85	977.81

Table 4.11. Average concentration of Lum in DBS filters collected from Central

	DAY 0	DAY 3	DAY 14	DAY 28
Subjects Code	**Mean Concentration (mg/L), (n=3)**	**Mean Concentration (mg/L), (n=3)**	**Mean Concentration (mg/L), (n=3)**	**Mean Concentration (mg/L), (n=3)**
085	1316.69	1138.65	957.94	610.56
167	1146.38	1057.72	649.52	627.48
171	1144.57	1315.13	603.03	609.25

Log concentration versus time for two of the subjects were plotted to mimic those that are used to adduce pharmacokinetic profiles. These are shown in Figure 4.6 A and B. As seen from the figures, the data satisfied the requirements for adducing this kind of data if a complete set of baseline data was available. This confirms that this method is capable of producing data that can be used to study the PK profiles of Lum in patients afflicted with malaria. This method is also good for performing therapeutic drug monitoring (TDM) especially in infants with uncomplicated plasmodium *falciparum*.

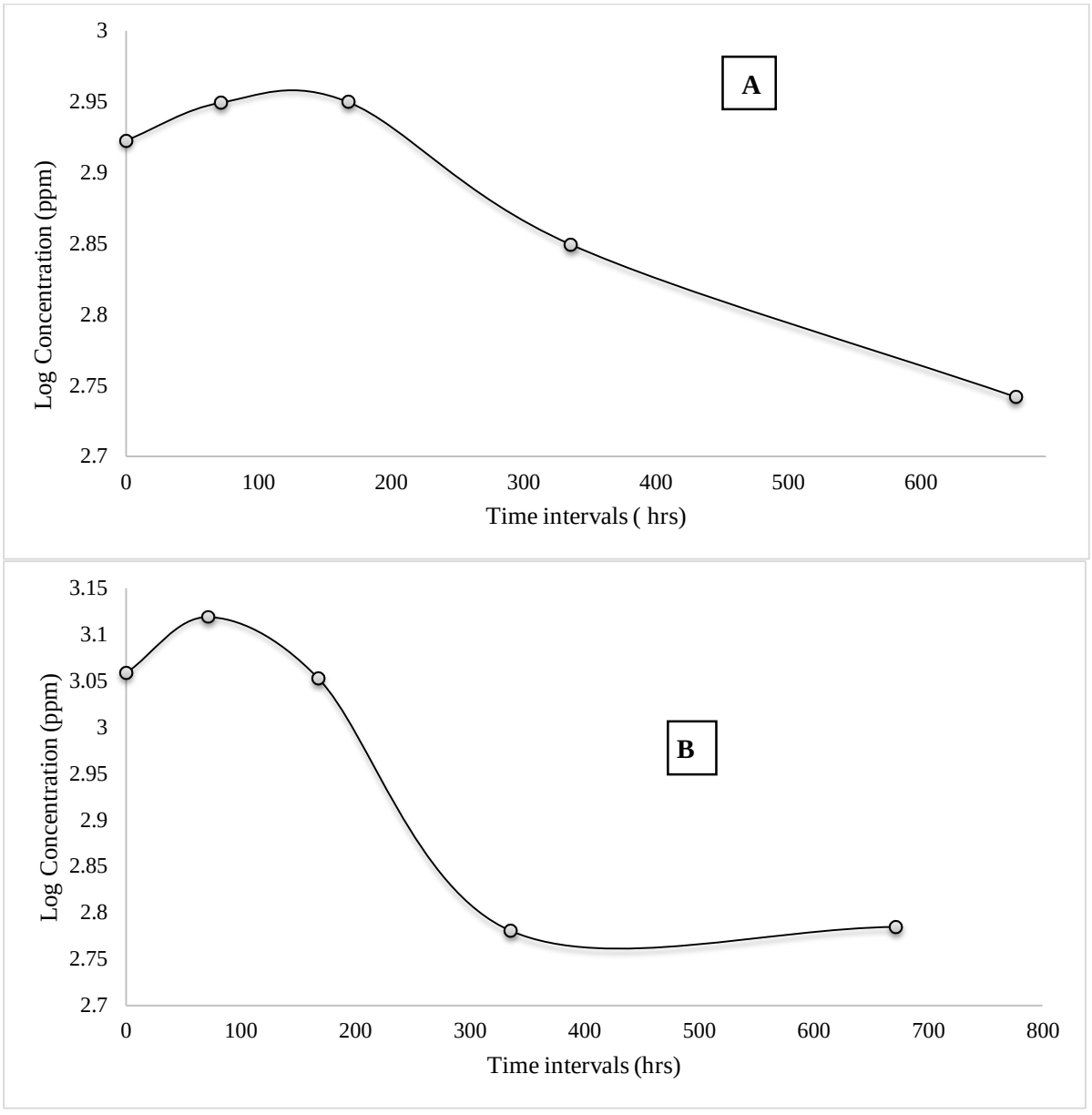


Figure 4.6. Logarithmic concentration-time graph over 28 days period for patient from (A) Ngamiland and (B) Central district receiving fixed artemether-lumefantrine dose containing 120 mg of lumefantrine.

From the graphs the concentrations increased and reached a peak before they dropped. This is an expected phenomenon in PK and TDM studies. It can also be seen that the maximum concentrations with time from the graphs were different after dosing the patients with Lum. This method can be used by biomedical and medical professionals to apply to their relevant fields.

Table 4.12. Comparison of data from this study with literature values

Sample type	Method	Extraction method	LOD (ng/mL)	LOQ (ng/mL)	% recovery	Reference
Human plasma	LC-MS/MS	PPT	5.3	21	< 30	Silva *et al.,* (2015)
DBS	LC-UV	LLE	-	158.7	45-51	Ntale *et al.,* (2008)
Plasma	LC-UV	Automated SPE	-	24	63-75	Lindegårdh *et al.*, (2005)
Human Plasma	LC-MS/MS	PPT	-	200	93.16	Pingale and Mangaonkar., 2013
DBS	LC-UV	SLE	400	800	61.7-123.9	This study

The non-specificity of LC-UV can be seen in the differences of the LOD and LOQ values estimated in this study compared to LC-MS methods. However, the percent recoveries obtained in this study were orders of magnitude better than in many other studies due to the simplicity of the extraction method on relatively clean sample collection media. The applicability was also justified due to the high therapeutic range of lumefantrine in both malaria free and malaria infected subjects which averages around 1- 10000 ng/mL (Silva *et al*, 2015; Huang *et al*, 2010; Ntale *et al*., 2008).

CHAPTER FOUR

5.0 Conclusions

A LC-DAD method for the quantification of lumefantrine in human samples collected on dried blood spot filters was successfully developed and validated using the United States Food and Drug Administration (USFDA) guidelines. This method satisfied the guidelines and was applied in quantification of lumefantrine in real DBSs filters from twelve subject from Botswana treated for malaria. This method is applicable in TDM and PK studies. To the best of the literature search carried out during this study, this forms the first study to avail this kind of data in Botswana.

5.1 Recommendations

Future research work on developing analytical extraction techniques which are solvent efficient and environmentally friendly should be done to evaluate the extraction efficiencies of both traditional and modern techniques using DBS filters for sample collection. Further work on developing an analytical method that can quantify artemether and lumefantrine simultaneously in DBS filters should be done to cover for routine therapeutic drug monitoring procedures.

5.2 Limitations

Due to excessively high cost of pure standards of artemether, it was not possible to quantify both lumefantrine and artemether simultaneously, compromising the initial desire of the project. Further, the half-life of artemether is extremely short which complicated the initial optimization experiments which were based on the use of a technical grade standard. It was decided to focus the attention on lumefantrine for these reasons.

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MINISTRY OF HEALTH
PRIVATE BAG 0038
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REF: DPH 20/4 XIII (99)

7th February, 2017

Research and Development
University of Botswana
P/Bag 0022
Gaborone Botswana

Dear Sir,

DRUG THERAPEUTIC EFFICACY MONITORING ON THE TREATMENT OF MALARIA CONFIRMED CASES

This correspondence serves to confirm that the School of Medicine is conducting the above study in partnership with the Ministry of Health and Wellness in line with the WHO recommendation that all malaria cases will be treated and follow-up for 28 days in order to ensure that parasitological cure is achieved.

The objective of the study is to monitor for treatment failure as well as to make a distinction between recrudescence and re-infection as prescribed in the Guidelines for the Diagnosis and Treatment of Malaria in Botswana (July, 2015). Samples are derived from Dried Blood Spots (DBS) on PCR paper obtained from malaria confirmed patients who visit Health Facilities from whom consent has been granted. The DBS is prepared concurrently with the Malaria Parasite Slides for microscopy and the Rapid Diagnostic Tests which are read immediately to inform case management while the DBS is sent to the School of Medicine due to challenges with PCR capacity at the National Health Laboratory.

The sample analysis is carried out by undergraduate students and Laboratory Scientists from the National Health laboratory under the supervision of Dr. Elias Peloewetse (Department of Biological Sciences) and Dr. Kwenga Sichilongo (Department of Chemistry).

Yours sincerely,

T. Moswcunyane
FOR/ACTING DIRECTOR PUBLIC HEALTH

UNIVERSITY OF BOTSWANA

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Ref: UBR/RES/IRB/BIO/GRAD/007

29th March 2017

Mr Blondie O. Brooks
Department of Chemistry
Faculty of Science
University of Botswana

RE: "PHARMACOKINETIC STUDY OF ARTEMETHER-LUMEFANTRINE FOR UNCOMPLICATED PLASMODIUM FALCIPARUM INFECTION IN THREE MALARIA-ENDEMIC DISTRICTS OF BOTSWANA"

Dear Mr Brooks,

I am pleased to inform you that the above mentioned study was reviewed and approved by the University of Botswana Institutional Review Board (UB IRB).

- APPROVAL DATE : 29th March 2017
- EXPIRATION DATE : This approval expires on 29th March 2018

  After this date, this project may only continue upon renewal. For purposes of renewal, a progress report should be submitted to ORD one month before the expiration date.

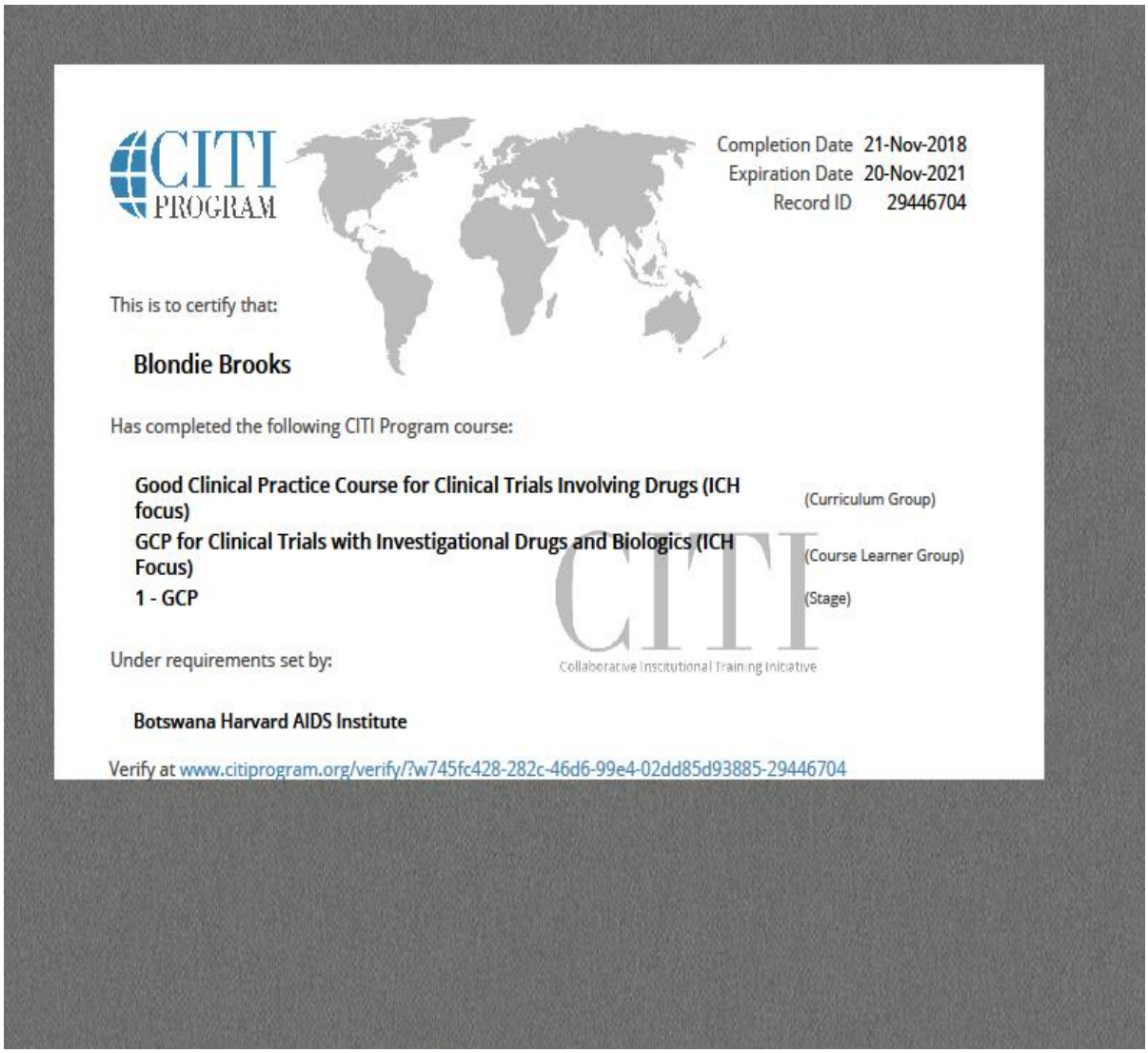
- REPORTING OF SERIOUS PROBLEMS: All serious problems impacting on study quality and progress (whether expected or unexpected) must be reported to ORD within 10 days.
- MODIFICATIONS: Prior approval is required before implementing any significant changes to the protocol.
- TERMINATION OF STUDY: On termination of this study, a report has to be submitted to ORD.
- **QUESTIONS**: Please contact ORD telephone (+267) 3552911 or e-mail on mary.kasule@mopipi.ub.bw.

- **Other:**
  - **The researcher may accordingly proceed with the above study in compliance with the above requirements.**

Kind regards.

UNIVERSITY OF BOTSWANA Office of Research & Development 2017 -04- 05 PRIVATE BAG 00708 GABORONE TELEPHONE: +267 3552900 FAX: +267 3957573 E-mail: research@mopipi.ub.bw

Dr M. Kasule
Assistant Director Research Ethics, Office of Research and Development



CITI PROGRAM

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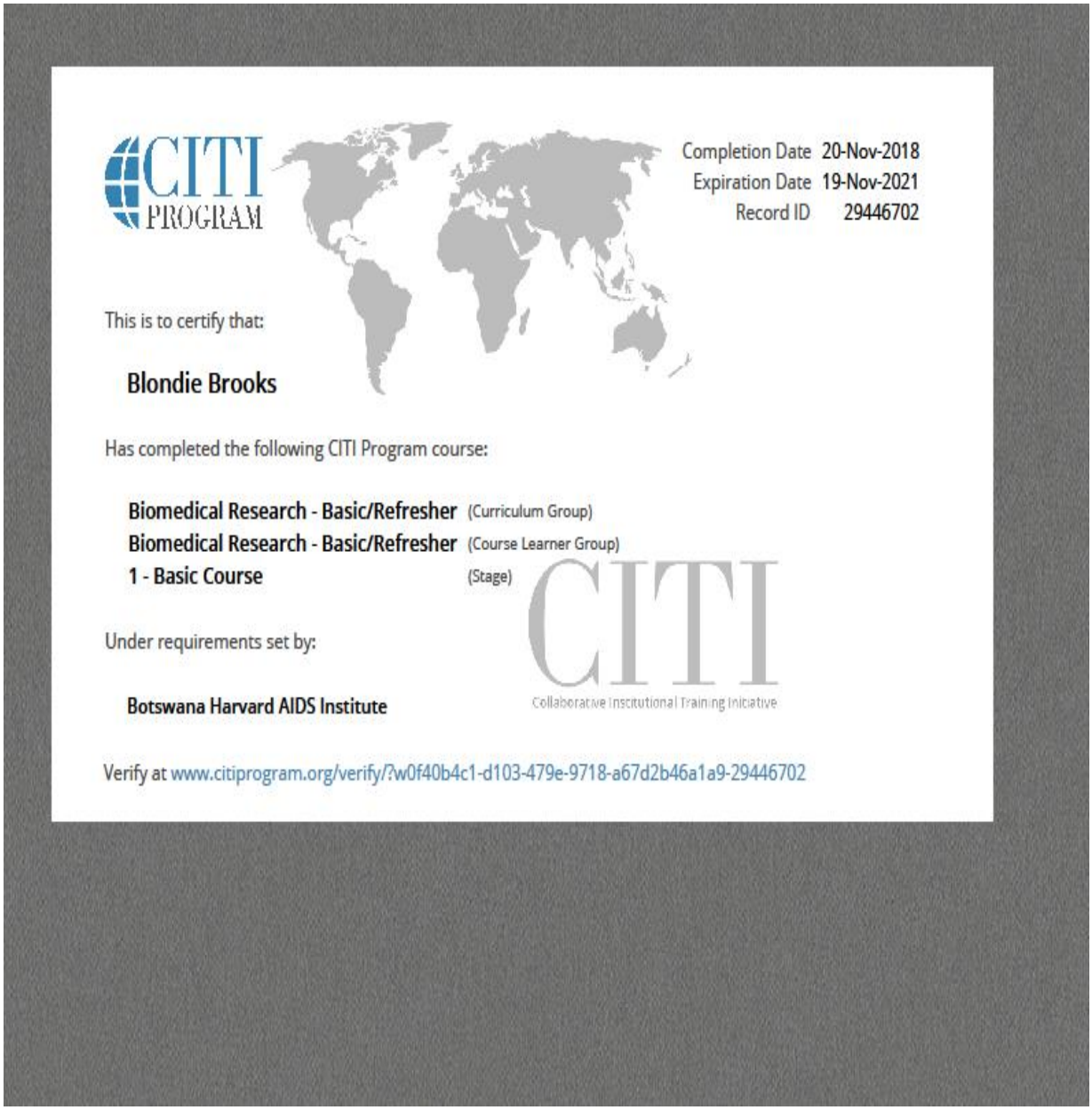

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Expiration Date 19-Nov-2021
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