LIÈGE université
Center for Interdisciplinary Research on Medicines

Faculty of Medicine
Department of Pharmacy
Laboratory of Pharmaceutical Analytical Chemistry
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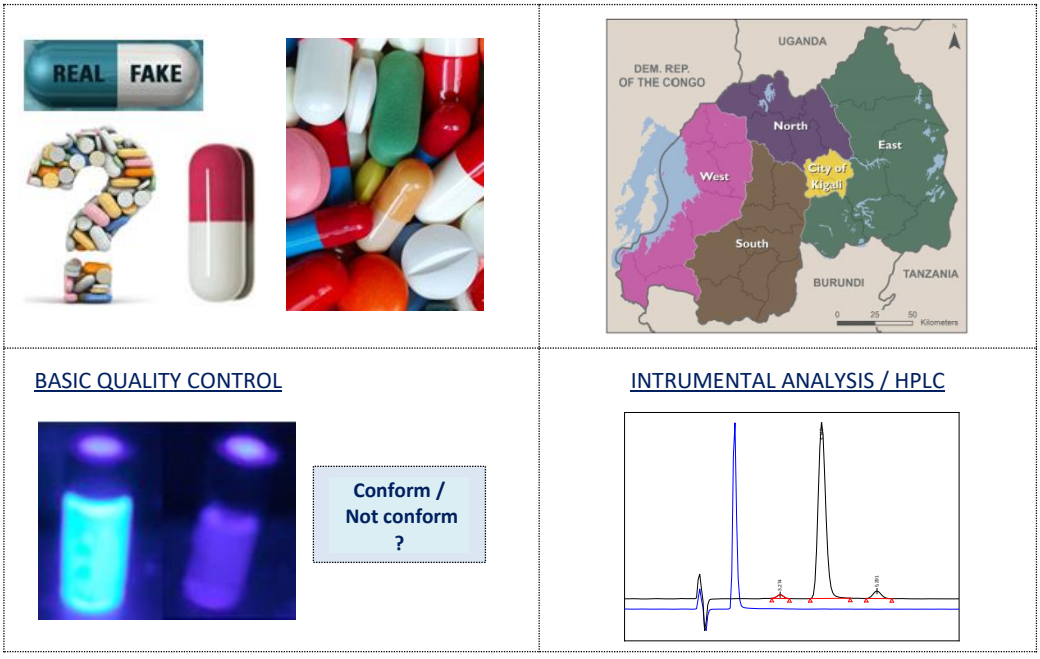
DEVELOPMENT, VALIDATION, AND TRANSFER OF GENERIC ANALYTICAL METHODS FOR FIGHTING AGAINST COUNTERFEIT MEDICINES



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" A hundred times a day I remind myself that my inner and outer life depend on the labors of other men, living and dead, and that I must exert myself in order to give in the measure as I have received and am still receiving "

- Albert Einstein -

……

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Abbreviations

	A
AIDS	Acquired Immune Deficiency Syndrome
API	Active Pharmaceutical Ingredient
ARV	Antiretroviral medicine
	B
BUFMAR	Bureau des Formations Médicales Agréés du Rwanda
	C
cf.	Confer or compare
cGMPs	Current Good Manufacturing Practices
CHWs	Community Health Workers
CPDS	Coordinated Procurement and Distribution System
	D
DAD	Diode Array Detector
DALY	Disability-Adjusted Life Year
DART	Direct Analysis in Real Time
DoE	Design of Experiments
DoE-DS	Design of Experiments & Design Space
DP	District Pharmacy
DRC	Democratic Republic of the Congo
DS	Design Space
	E
EAC	East African Community
	F
FDA	Food and Drugs Authority
	G
GAPs	Good Auditing Practices
GCPs	Good Clinical Practices
GDPs	Good Distribution Practices
GxPs	Good Practices
	H
HF	Health Facility
HIV	Human Immunodeficiency Virus infection
HPLC	High Performance Liquid Chromatography
HSSP	Health Sector Strategic Plan
	I
ICH	International Conference on Harmonisation
ICT	Information and Communication Technology
IHME	Institute for Health Metrics and Evaluation
	J-K
-	-

	L
LABOPHAR	Laboratoire Pharmaceutique du Rwanda
LADAMET	Laboratoire d'Analyse des Denrées Alimentaires, Médicaments, Eaux et Toxiques
LC	Liquid Chromatography
LOD	Limit of detection
LOQ	Limit of quantitation
	M
m/z	Mass-to-charge ratio
MDGs	Millennium Development Goals
MoH	Ministry of Health
MPPD	Medical Procurement and Production Division
MS	Mass Spectrometry
	N
NMR	Nuclear Magnetic Resonance
NMRA	National Medicines Regulatory Authority
NQCL	National Quality Control Laboratory
NRL	National Reference Laboratory
NSAI	Nonsteroidal anti-inflammatory
	O
OOS	Out-of-Specification
	P
p.	Page
ppm	Parts per million
PQM	Poor Quality Medicine
PT	Proficiency Testing
PTB	National Metrology Institute of Germany (Physikalisch-Technische Bundesanstalt)
	Q
QA	Quality Assurance
QAQC	Quality Assurance and Quality Control
QC	Quality Control
	R
R&D	Research and Development
RBC	Rwanda Biomedical Center
RBC/MPPD	RBC/Medical Procurement and Production Division
RNEC	Rwanda National Ethics Committee
RSB	Rwanda Standards Board
RSD	Relative standard deviation
	S
SAMU	Service d'Aide Médicale Urgente (i.e. Emergency Medical Services)
SCMS	Supply Chain Management System
SD	Standard deviation
SDGs	Sustainable Development Goals
SDP	Service Delivery Point
SF	Substandard and Falsified
SFSTP	Société Française des Sciences et Techniques Pharmaceutiques
SSFFC	Substandard / Spurious / Falsely-labelled / Falsified / Counterfeit

	T
TB	Tuberculosis
	U
ULg	University of Liège
UN	United Nations
UR	University of Rwanda
USA	United States of America
USD ($)	United States Dollar
USP	United States Pharmacopeia
UV	Ultraviolet
	V
vs	versus
	W
WHO	World Health Organization
	X
-	-
	Y
YLD	Year(s) Lost due to Disability
YLL	Years of Life Lost due to premature mortality
	Z
-	-

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PART I - INTRODUCTION

I.1 Overview on Counterfeit Medicines and related consequences to the Public Health

I.1.1 Introduction to poor quality medicines

According to the Cotonou declaration against fake medicines, the criminal economy of counterfeit medicines is appalling [1]:

- Because it preys on the poorest countries, and within them, on families with no social protection and no financial means;
- Because it involves drugs that are the most vital to individual and public health: treatment for malaria, tuberculosis, and AIDS;
- Because it has spread everywhere, into street markets, on the internet, and because it has developed to the point of becoming almost more profitable than the illegal drug trade;
- Because counterfeit medication does not simply dupe the hopes of the ill; they are poisons that often kill or handicap.

Indeed, the presence of poor quality medicines (see definition latter) on the market constitutes a harmful threat to the Public Health worldwide and particularly in developing countries where drug regulatory systems are often weak and therefore cannot ensure good quality of medicines on their territories that can seriously affect the efficacy and safety. On the other hand, estimations put counterfeit medicines at more than 10% of the global market, and they are present in all regions especially in under-resourced countries where they can reach 30% or even more alarming proportions associated to serious consequences such as treatment failures, development of drug resistance, adverse effects, mortality and morbidity, economic loss for patients, families and even governments, etc. [2-3]. Hence, there is need of developing simple, rapid, effective, and affordable analytical tools that can be used in drug quality control laboratories and proficiently help in detecting and fighting against the spread of poor quality medicines.

The circulation of poor quality medicines constitutes a serious threat to the public health and socio-economic life worldwide especially in developing countries where the manufacturing,

importation, distribution, supply and sales of medicines are less regulated and poorly monitored [4-10].

Moreover, any kind of medicine and medical devices can be and has been counterfeited such as expensive lifestyle and anticancer medicines, antibiotics, medicines for hypertension and cholesterol-lowering medicines, hormones, steroids and inexpensive generic versions of simple pain killers and antihistamines, blood glucose test strips and condoms, etc. In developing countries the most disturbing issue is the common availability of counterfeited medicines for the treatment of life-threatening conditions such as malaria, tuberculosis and HIV/AIDS [4].

As illustrated in **Figure 1**, poor quality medicines can be classified into three main categories: counterfeit/falsified, substandard, and degraded medicines [5].

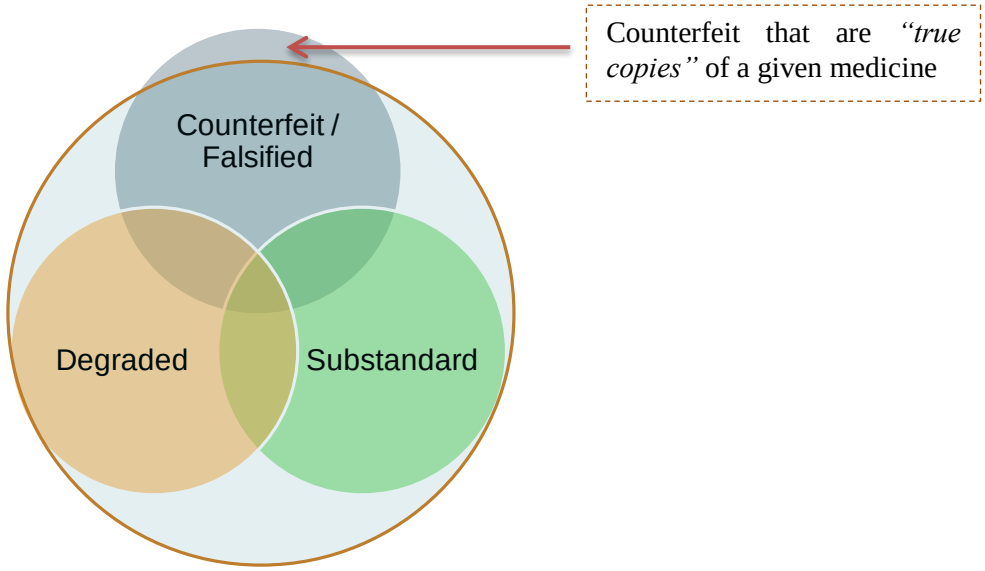


Figure 1 *Categorization of poor quality medicines (adapted from Paul N. Newton et al.)*

Note: There could be grey areas between all three main types. For example, both substandard medicines and counterfeits can degrade after manufacture; and not all counterfeit medicines are out-of-specifications of the required quality standards as there are counterfeit organizations that have sophisticated production capacities for manufacturing products and labeling strikingly similar to the authentic products [5-6].

The category of *"counterfeit or falsified"* medicines is defined as products deliberately and fraudulently produced without any regard to regulatory and public health concerns.

The category of *"Substandard medicines"* also called *out-of-specification (OOS) products* are genuine medicines produced by manufacturers authorized by the NMRA which do not meet quality specifications set for them by national standards.

The category of *"Degraded medicines"* sometimes associated with substandard is for those which are spoiled and lost their original quality due to poor handling (storage conditions) or expiration.

Hence, from the above definitions, poor quality medicines may include [4; 9]:

1. Products containing correct or wrong ingredients;
2. Products containing overdose, low dose or no active ingredients;
3. Products with fake packaging and labelling;
4. Products with poor stability;
5. Products with unsatisfactory dissolution profiles;
6. Expired medicines relabeled with the purpose to extend the shelf-life;
7. Products without the name and address of the manufacturer;
8. Expired products;
9. Products with no expiry date;
10. Products affected by chemical or microbial contaminations containing higher level of impurities, microorganisms, etc.

On the other hand, the World Health Organization (WHO) defines counterfeit medicines as the "*one which is deliberately and fraudulently mislabeled with respect to identity and/or source. Counterfeiting can apply to both branded and generic products and counterfeit products may include products with the correct ingredients or with the wrong ingredients, without active ingredients, with insufficient active ingredients or with fake packaging"* [7].

Note that the WHO has updated in May 2017 the name of ***"substandard and falsified"*** (SF) medical products that will be used for what had been previously known as *"substandard/spurious/falsely-labelled/falsified/counterfeit (SSFFC)"* medical products. The

WHO also agreed a definition of ***"unregistered or unlicensed medical products"***. These have not been assessed or approved by the relevant national or regional regulatory authority for the market in which they are marketed, distributed or used [8].

The new terminology aims to establish a common understanding of what is meant by substandard and falsified medical products and to facilitate a more thorough and accurate comparison and analysis of data. It focuses solely on the public health implications of substandard and falsified products, and does not cover the protection of intellectual property rights.

I.1.2 Factors facilitating pharmaceutical counterfeiting

Easy Money is the main driver for counterfeiters as the manufacturing costs are very low in clandestine places where no quality and safety standards are respected. Then, a variety of factors make drug counterfeiting possible [4; 7]:

- Thirst for **easy money** is the main driver for counterfeiters as manufacturing costs are very low for them due to non-respect of quality and safety standards;

- **Inadequate legislation, regulations and enforcement** result in supply systems vulnerable to counterfeit products and extremely low capacity to uncover and punish counterfeiters;

- **Ineffective cooperation among stakeholders:** health authorities, customs, police, industry and trade should establish effective cooperation and exchange of information in order to detect and stop counterfeiters;

- **Lack of awareness:** ignorance of the risks of counterfeit medicines among health professionals and patients hinders detection and reporting, even when patients face treatment failure or adverse reactions;

- **Costs of medical products:** the costs of legitimate medicines (both original and generics) may be too high for patients, and the later unable to afford the costs of

legitimate medicines can take high risk to go for cheaper medications in unregulated markets such as street markets or the Internet;

- **Lack of political will:** in some countries authorities are not prepared to recognize the existence of the problem or to pursue counterfeiters if there is inadequate appreciation of the public health value of medical products compared to considerations of export interests;

- **Weak penal sanctions:** absence of or lenient penal sanctions for violations of drugs legislation may encourage counterfeiting;

- **Corruption and conflicts of interest** may adversely affect the efficiency of drug regulatory authority and law enforcement personnel, resulting in a failure to arrest, prosecute and convict those responsible for counterfeiting;

- **Transactions involving many intermediaries** increase opportunities for counterfeiters to infiltrate the regulated distribution system;

- **Lack of regulation by exporting countries and within free trade zones:** Pharmaceuticals made "For Export" (sometimes labelled "For Export Only") are often not regulated by exporting countries to the same standards as those produced for domestic use. In addition, pharmaceuticals are sometimes exported through free trade zones where drug control is negligent and where repackaging and relabeling take place; this can facilitate trade in counterfeit medicines.

I.1.3 Extent of poor quality medicines

It is difficult to obtain precise figures on the extent of counterfeit medicines due to the lack of sufficient data across the World. The United States Food and Drug Administration estimates that counterfeit medicines represent about 10% of the global pharmaceutical market, while many developing countries of Africa, parts of Asia, and parts of Latin America have areas where more than 30% of the medicines on sale can be counterfeit [2-3; 10].

The map in **Figure 2** shows the global repartition of counterfeit medicines as per Sanofi-Aventis (2008) [11] and the status of the problem is still illustrative for nowadays, while **Figure 3** illustrates the geographical breakdown of pharmaceutical crime incidents in 2015.

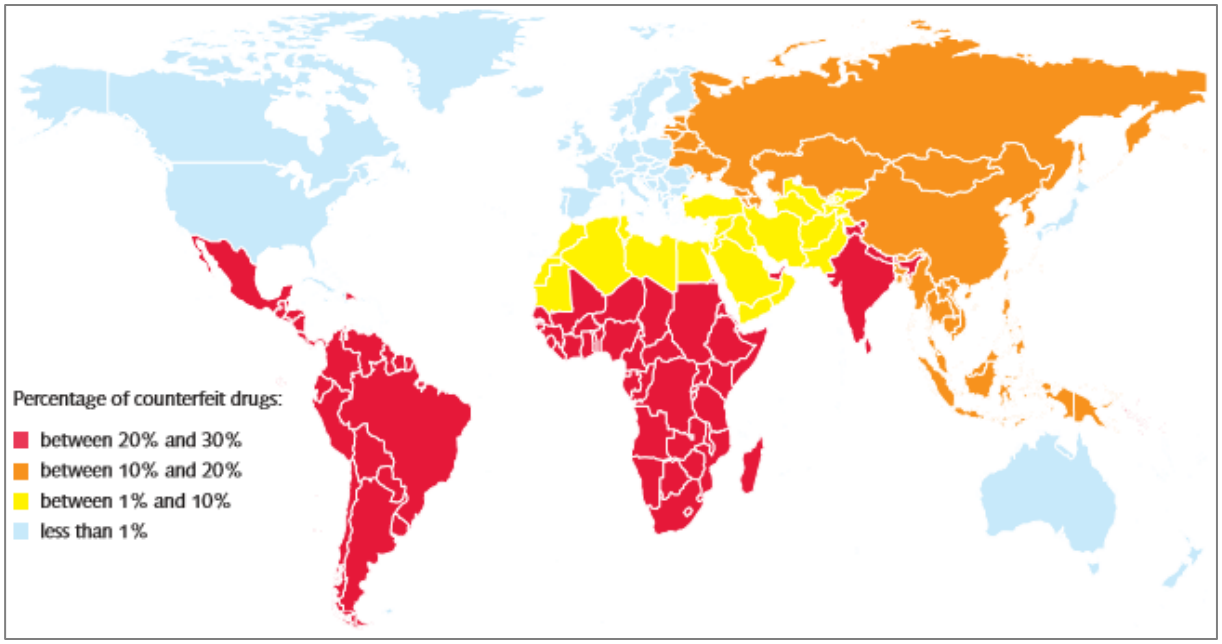


Figure 2 *Global Repartition of Counterfeit Medicines*
Source: Sanofi-Aventis (2008) [10]

Figure 3 *Geographical breakdown of pharmaceutical crime incidents in 2015*
Source: Pharmaceutical Security Institute, 2016 [11]

Note: An incident means that a region has been identified as the origin, point of seizure or transit, or destination of illegal pharmaceuticals, and one can notice that counterfeit medicines are everywhere in the world. However, note that having recorded few incidents in Africa, Middle East and Eurasia do not mean that those regions are the ones having fewer counterfeit/falsified products, but having simply fewer reported cases.

I.1.4 Consequences of poor quality medicines

The use of poor quality medicines can be harmful to the population at both medical and socio-economic aspects through therapeutic failures, drug resistance, loss of efficacy, adverse effects, loss of confidence in health systems and health workers, increased mortality and morbidity, infringement of intellectual property rights, economic loss for patients/their families/health systems/producers and traders of genuine products, etc. [2; 4; 6-8].

Moreover, note that in most cases, counterfeit drugs are not equivalent in quality, safety and efficacy to their genuine counterparts. Even if they seem to be of correct quality or contain the correct amount of active ingredients, their production and distribution are not within the purview of the drug regulatory authority of the country concerned. This means that any associated defects and adverse reactions will not be easily recognized or monitored and, if needed, an effective product recall would not be possible [7].

I.2 Overview on medicines Quality Assurance in Rwanda

I.2.1 Country profile

Rwanda is located in the Central / Eastern Africa at 1,200 km from the Indian Ocean to the East and 2,000 km from the Atlantic Ocean to the West. The surface area of 26,338 km^2 is 930 km bordered by Uganda to the North with 172 km of border length, Tanzania to the East with 222 km border length, the Democratic Republic of the Congo (DRC) to the West with 221 km border length, and Burundi to the South with 315 km border length as shown in the country map (**Figure 4**), and some other useful information are given in **Table 1** [12-16].

Figure 4 *Map of Rwanda*

Source: World Health Organization [12]

Table 1 *Useful data on Rwanda*

Indicators		**Statistics (2013)** [15]	**Latest (May 2018)** [16]
Population		11,777,000	12,501,156
Population density (people / km^2)		447	507
Population aged under 15 years (%)		43	-
Population aged over 60 years (%)		4	-
Median age (years)		18	19.6
Population living in urban areas (%)		27	34.0
Total fertility rate (per woman)		4.5	4.1
Population growth rate (average annual %)		-	2.4
Life expectancy	At birth	65	-
	At age 60	18	-
	Females	-	69.3*
	Males	-	66.0*
World bank income classification		Low	-

* Year: 2016 (cf. Reference [17])

On the other hand, according to the Institute for Health Metrics and Evaluation (IHME, Seattle, Washington / USA), the top 10 causes of disability and death combined commonly known as disability-adjusted life years (DALYs) for Rwanda in 2016 are shown in **Figure 5**

where we can notice that Malaria and HIV/AIDS epidemics are respectively 5th and 6th in the ranking [17]; and related medicines were selected for study in the frame of this thesis.

To understand the figure, a DALY can be thought of as "one lost year of a healthy life", and DALYs are used as unit of measurement that combines information about morbidity and mortality. For example, ten DALYs correspond to ten lost years of healthy life, attributable to morbidity, mortality, or both. Then, DALYs for a disease or health condition are calculated as the sum of the Years of Life Lost (YLL) due to premature mortality in the population and the Years Lost due to Disability (YLD) for people living with the health condition or its consequences [18-19]. In the figure, the red bars indicate the level of percent changes between 2005 and 2016 per disease; and the negative levels such as -10%, -30%, -50%, etc. indicate that the disability-adjusted life years have decreased at those levels, often due to the improvement of existing health programs in disease control and prevention, while positive changes such as 10%, 20%, 30%, etc. indicate the increase of DALYs due to more morbidity and/or mortality that occurred in the studied period.

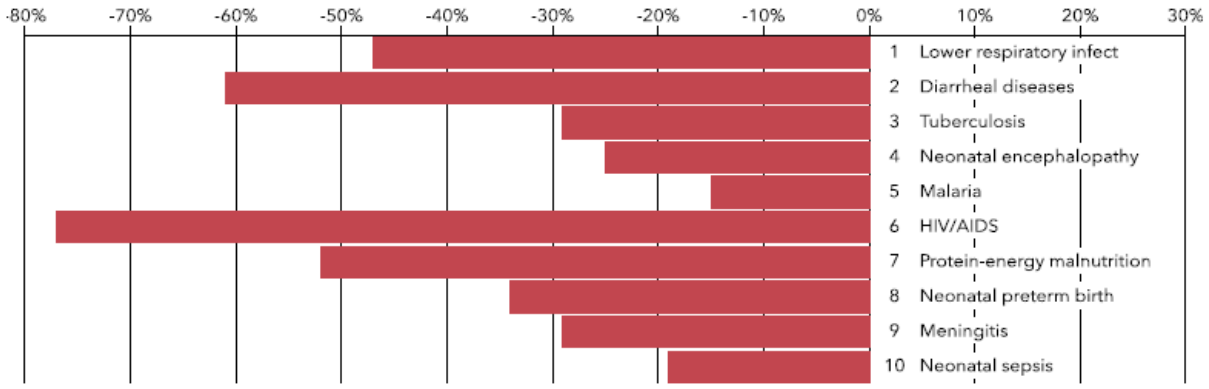


Figure 5 *Top 10 causes of death and disability combined (DALYs) in Rwanda and percent change between 2005-2016, all ages* [17]

In fact, we selected antimalarial and ARV medicines in our study due to the high priority the two epidemics are given in the Rwandan Health Sector Strategic Plans (HSSP - I, II, and III) from 2005 to 2018 aligned with the United Nations (UN)-Millennium Development Goals (MDGs, 2000-2015), Africa Health Strategy (2007-2015), UN-Sustainable Development Goals (SDGs, 2016-2030), etc. [18-21]. Therefore, eight ambitious MDGs were internationally committed on, aiming to:

- Eradicate extreme poverty and hunger (MDG 1)
- Achieve universal primary education (MDG 2)
- Promote gender equality and empower women (MDG 3)
- Reduce child mortality (MDG 4)
- Improve maternal health (MDG 5)
- Combat HIV/AIDS, malaria and other diseases (MDG 6)
- Ensure environmental sustainability (MDG 7), and
- Develop a global partnership for development (MDG 8)

Among them, the underlined MDGs (1, 4, 5, and 6) are directly linked to health, and the MDG 6 is specific to "Combatting HIV/AIDS, malaria, and other diseases".

On the other hand, after the UN MDGs period, the latter were replaced by SDGs oriented to seventeen goals around five core themes: people, planet, prosperity, peace, and partnership. Thus far, in contrast to the MDGs, which were primary focused on progress in developing countries; the SDGs aim to support and promote well-being for everyone, and everywhere in developing and developed countries. Hence, the consolidated health goal (SDG 3) is at the core of the SDG framework, closely linked to each and every other goal. The SDG 3 is to *"Ensure healthy lives and promote well-being for all at all ages"*; for which 13 targets are defined including the target for infectious diseases stipulating that "By 2030, end the epidemics of AIDS, tuberculosis, malaria and neglected tropical diseases and combat hepatitis, water-borne diseases and other communicable diseases" [18].

I.2.2 Rwanda supply chain network description

Different levels and institutions from public and private sectors are actively involved in the network of the supply chain of medicines in Rwanda. The national level supports district pharmacies (DPs) and service delivery points (SDPs) to increase access to healthcare commodities to clients / patients.

The private sector complements the public sector for the supply of commodities, even though the public sector provides the majority of commodities. On the other hand, the information

flow through the network provides data and feedback useful for decision making at all levels of the supply chain as illustrated in **Figure 6** [22].

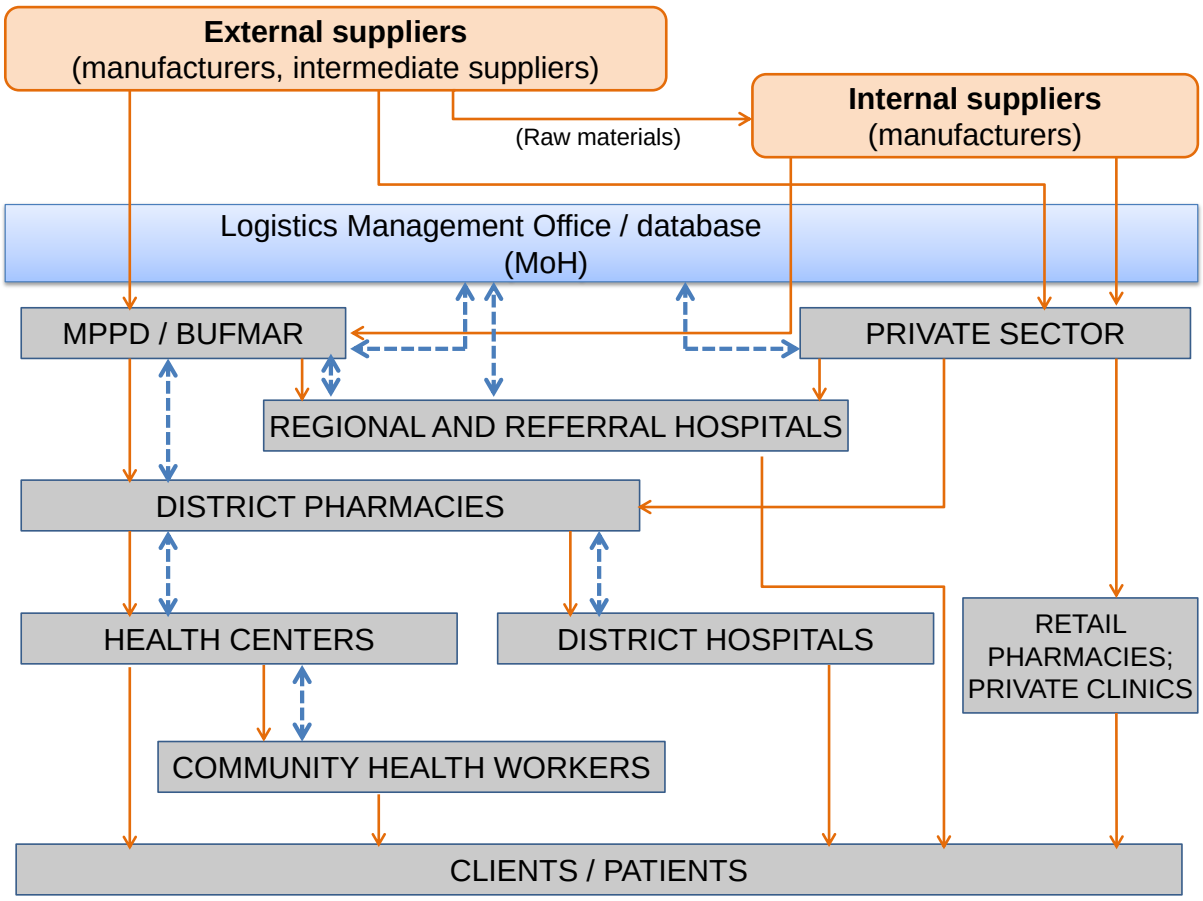
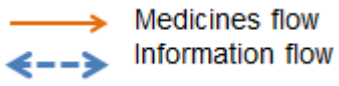


Figure 6 *Rwanda supply chain network (adapted from the Supply Chain Management System "SCMS" / Rwanda Ministry of Health*) [22]

Legend:



Such complex supply chain needs appropriate measures including control strategies in terms of products "sanctity" through inviolability or security labels, "identification and traceability" through barcoding or numbering of labels, data matrix or serialization / products information in time and space, and "authentication" through verification of products per internet or mobile phone short messages to confirm the authenticity in order to ensure that the products on market are good and genuine.

It should be noted that today almost all medicines used in Rwanda are imported by both public and private sector generally providing medicines, medical consumables, medical devices and equipment, laboratory supplies, food supplements and other pharmaceutical services needed in the country The imported products come from several countries from the region such as Uganda, Kenya, South Africa, Mauritius Island, etc., and mostly from countries outside the continent such as India and China in Asia; Belgium, Germany, Switzerland in Europe; and USA, etc.. In fact, the Rwandan market share of locally manufactured pharmaceutical drugs is estimated to be less than 1% according to the Federation of East African Pharmaceutical Manufacturers Association Chairman [23], but the Government of Rwanda is continuously inviting and encouraging investors in local pharmaceutical manufacturing. In this context, two big projects have been launched by Cooper Pharma (a leading African pharmaceutical firm based in Morocco) and Apex Biotech Ltd (a co-owned pharmaceutical firm by Rwandans and Bangladeshi investors). Cooper Pharma has launched the construction works of a $USD 6 million plant in January this year for the production of beta-lactam antibiotics in the short term, and then will expand to non-beta lactam antibiotics depending on the local and regional market demands; the factory is expected to be completed in 2019 [24]. On the other hand, the Apex Biotech Ltd project worth $USD 18 million is expected to open next year too, and the plant will manufacture generic pharmaceutical formulations in all major therapeutics in a variety of dosage forms including tablets, capsules, powder for suspension, liquid syrups, and oral rehydration therapy [25].

Note that in Rwanda, procurement functions of medicines are mainly fulfilled by the Central Medical Store “currently RBC/MPPD”, Referral hospitals, BUFMAR, and Private pharmacy wholesalers. Then, the supply chain network across the country can be divided into five core hierarchical levels: (i) National, (ii) District, (iii) Health centers, (iv) Health posts and (v) Community. For the private sector, there are two levels: (i) Wholesalers, and (ii) Retail pharmacies.

Further additional information on different administrative structures and related health facilities involved in the supply chain of medicines, approximate numbers of population per category, etc. are summarized in the following table.

Table 2 *Existing administrative structures and related health facilities, HSSP 4 (2018)* [26-27]

Administrative level	Number	Health care delivery system	Health care Category	Number of health facilities (HFs) per category	Average catchment area population
National: (MoH, etc.)	1	National referral & Teaching hospitals	Tertiary	8	≈ 12 millions
Province & Kigali	5	Provincial hosp.		4	-
District	30	- District hospitals - District Pharmacies	Secondary	36 30	≈ 255,000
Sector	416	Health centers	Primary	499	≈ 23,000
Cell	2,148	Health posts (*)		476	-
Village	14,837	CHWs	First aid	45,516	≈ 250
Household	-	Family aid	Beneficiary	-	-
Other structures: SAMU services Private HFs	 - -	 Ambulances Private sector	 Emergency -	 225 ambulances 250 registered	 - -

I.2.3 Description of the Rwandan medicines quality assurance system based on the national pharmacy policy

I.2.3.1 Introduction

In Rwanda, availing safe, effective and high quality medicines, health commodities, and technologies that are accessible to the population has always been a concern of the Government. Thus, since 1996, the Government of Rwanda has undertaken a series of quality improvement interventions and has held numerous discussions on how to meet and uphold the standards to make essential, high-quality health commodities and technologies accessible to all residents, in accordance with the national Pharmacy, and Health Sector policies [28-29].

Then, to fulfil its mission to provide quality healthcare for all its population, the Government of Rwanda has always taken bold steps in its health policy, such as the Alma Ata Declaration of 1978 on "expressing the need for urgent action by all governments, all health and development workers, and the world community to protect and promote the health of all the people of the world" and the Bamako initiative of 1987 sought to accelerate and strengthen the implementation of primary healthcare, with the goal of achieving universal accessibility to these services. It was an initiative that African Ministers of Health introduced in 1987 to reorganize and strengthen primary healthcare with emphasis on maternal and child health.

Hence, by adopting the *National List of Essential Medicines*, creating a *Pharmaceutical Service Unit* within the Ministry of Health, and establishing a *Central Medical Store* for essential medicines, commodities, and technologies and allowing *Exemption from entry duties* on these items, Rwanda is boldly moving forward to develop its pharmaceutical sector so that quality healthcare can be available to its population.

I.2.3.2 Situation of the Pharmaceutical sector

The status of key components of the Rwandan pharmaceutical sector is presented below according to the national Pharmacy, and Health Sector policies referred to above.

A) Quality assurance

Quality assurance of medicines refers to all the necessary measures taken to ensure that products quality is maintained from the point of production to the point of use. Rwanda has established quality assurance systems to ensure that health commodities and technologies circulating in the supply chain meet national and international standards of quality, safety and efficacy.

However, despite all these efforts, the quality assurance framework has limited capacity in terms of infrastructure, human and financial resources.

B) Legislation

Pharmaceutical laws and Ministerial orders have been initiated, developed and gazetted to ensure that pharmaceutical services are properly regulated. The following documents have been put in place:

1. Law determining “The Art of Healing”;
2. Law relating to the regulation and inspection of food and pharmaceutical products;
3. Law governing narcotic drugs, psychotropic substances and precursors in Rwanda;

4. Law establishing Rwanda Food and Medicines Authority and determining its mission, organization and functioning;
5. Law on organization, functioning and competence of the Council of Pharmacists.

These laws have to be harmonized with East African Communities.

C) Regulation

The Rwanda Pharmacy sector is regulated at two levels: (i) Medicines, other health commodities and technologies and (ii) Pharmacy professionals.

- Currently, the Pharmacy Department in the Ministry of health combines both the responsibilities of Policy formulation and Regulatory activities to provide minimum services related to product evaluation and registration, import and export control, licensing and inspection of pharmaceutical establishment, supply chain monitoring, pharmacovigilance and post-market surveillance, pharmaceutical products advertisement and promotion.

- A National Pharmacy Council was established by the law n^{o} 45/2012 of 14/01/2013 to regulate the pharmacy profession.

  These need to be reinforced with quality control system (suitable and reliable analytical methods) while waiting for strong national FDA system.

D) Rational use of Medicines, Health commodities and technologies

Rwanda has established various mechanisms to promote medicines information, prescribing and dispensing; including the development of treatment guidelines and protocols, the national formulary and a national essential medicine list with their subsequent periodic reviews. In addition, pharmacovigilance systems have been established at different levels.

However, despite of these initiatives, Rwanda faces a number of challenges, including limited adherence to good prescribing and dispensing practices, and low reporting of adverse

reactions. Also, there is inadequate awareness among the community on the rational use of medicines, and inadequate reference materials at the health facilities.

E) Selection of medicines

It is a process of identifying medicines to satisfy the health care needs of the majority of population.

Indeed, the choice of medicines depends on many factors such as the prevailing diseases, treatments guidelines, level of health care delivery and financial resources.

However, Rwanda encounters challenges in medicines and therapeutic committees not performing optimally due to limited capacity and competences in pharmaceutical management.

F) Procurement, storage and distribution of health commodities

These are key pillars of an effective health care system. In Rwanda, procurement functions are mainly fulfilled by the Central Medical Store currently Medical Procurement and Production Division (MPPD), Referral Hospitals, BUFMAR (Bureau des Formations Médicales Agréées du Rwanda, a faith-based organization) and private pharmacy wholesalers.

The Coordinated Procurement and Distribution System (CPDS) has improved the availability of HIV related health commodities and technologies.

For public procurement of health commodities and technologies, an international competitive bidding is used; and the national procurement regulation law and stringent regulation adopted by partners are used for the procurement processes.

However, the overall storage infrastructure and equipment for health commodities and technologies need improvement in terms of capacity, design, maintenance and security, transport and information management systems.

G) Local Manufacturers of Medicines

Despite difficulties in the development of local capacity for the production of essential medicines, Rwanda has made some efforts to create a comprehensive environmental for investment. Rwanda must continue to work through the national investment authorities in providing guidance and incentives to create manufacturing capacity for specific investment in the pharmaceutical industry.

So far, the country has only one pharmaceutical factory (former LABOPHAR) that produces some essential medicines covering less than 2% of the needs of the country, and this puts the country on a high risk of procuring counterfeit medicines from illegal manufacturers.

H) Affordability

Currently there is no Pharmaceutical Pricing Policy both in public and private sector; hence this may render the prices of health commodities and technologies to be generally high and less affordable to the majority of the population. However, in the absence of the pricing policy, the Ministry of Health in collaboration with stakeholders established tariffs of services rendered in public health facilities**.**

In this situation, the risk of having very cheap and poor quality medicines is high when prices on the market are not well regulated and controlled. Hence, there is need to establish an efficient quality control system to regularly assess the quality of different vital medicines at reception in customs and after distribution to the community (post market surveillance).

I) Health product financing

The Government guarantee and commitment to an appropriate level of sustainable financing is an essential element in the success of the entire health sector and in particular, the pharmaceutical sub sector.

In Rwanda health commodities and technologies are financed through fiscal budgetary allocations, development partners, insurance schemes, private sector and out of pocket expenditure.

The population covered by any health insurance scheme is 96% (Rwanda health insurance indicators: progress 2010).

J) Pharmaceutical Research and Development

Research and development plays a major role in industrial transformation and economic growth, as well as in health care delivery. Pharmaceutical R&D is driven by current developments in disease control and treatment, in particular the need for enhanced use of diagnostics; early onset of treatment and use of newer, safe, more efficacious and age-appropriate medicines.

The pharmaceutical services in the Ministry of Health regulate clinical trials, in collaboration with the Rwanda Ethic committee (RNEC).

The School of pharmacy at the University of Rwanda and other research institutes have been undertaking various forms of research on medicines and treatment, including clinical trials and product development.

However, despite Rwanda is endowed with many medicinal flora and fauna there is very limited investment to exploit this endowment in making products for use in health care. In case where research has shown the presence of pharmacological activity in plants, the research ends in only publications but not in products development.

Hence, there is a need to increase and prioritize funding for pharmaceutical research and development activities that address local needs and attract investment.

K) Information and communication Technology (ICT)

There have been numerous advances in the field of ICT, and many of these have been embraced in various facets of the pharmaceutical sector. ICT use in pharmaceutical includes technological advancements in design and automation of processes, including procurement and logistics management, dispensing of health commodities, online reporting on medicines

safety and quality monitoring, availability and consumption of health commodities and technologies.

The country has made significant strides towards narrowing the digital divide within and the rest of the world, through infrastructure expansion and upgrading, and through an enabling legal and institutional framework. However, there remain some gaps in infrastructural and human resource capacity for the full application of ICT in the pharmaceutical sector.

Challenges relating to ICT in the pharmaceutical sector include:

a) Insufficient infrastructure, equipment, and skilled human resource in ICT for improved efficiency and effectiveness of pharmaceutical services;
b) Absence of ICT in regulatory functions (e.g. electronic submission of dossiers for registration of medicines, importation licenses, licensing of pharmacies). Currently all importation licenses are requested electronically.

To address the challenges of information technology in pharmaceutical sector, the Government is investing in ICT infrastructure for effective operation of public pharmaceutical services, including procurement (e-procurement), distribution, regulation and quality control. This is also evidenced in the regional initiatives for the pharmaceutical regulatory harmonization and integration.

On the other hand, there is need to ensure the Data Integrity and compliance with the good pharmaceutical practices (GxPs) such as the current good manufacturing practices (cGMPs), good distribution practices (GDPs), good clinical practices (GCPs), good auditing practices (GAPs), etc. In fact, the role of IT in the health sector has a tremendous positive impact in improving quality, safety, and efficiency/productivity. However, such computerized systems should be well managed and protected against any misuse, hacking, or dysfunctioning. The data integrity should also be assured by ensuring that they are updated, attributable to the authorized staff (or officers) for recording or editing, legible, contemporaneously recorded, original or true copy, and accurate.

L) Human Resource for the pharmaceutical sector

Rwanda recognizes the insufficient numbers of appropriately trained human resources in the pharmaceutical sector, and the existing gaps are increasing as demand increases.

In fact, the pharmaceutical work force in Rwanda includes pharmacists and pharmacy technicians and of the available 500 pharmacists, only 39% (i.e. around 200 pharmacists) are serving in the public sector, and 40% of those in the public sector (i.e. around 80 pharmacists) are employed in Kigali City while 60% are in other provinces. On the other hand pharmacists employed in the private sector (i.e. around 300 pharmacists), 75% are in Kigali City (i.e. around 230) while 25% are in other provinces. These figures show a disproportionate repartition of pharmacists in Kigali City, where more than 60% of the working pharmacists are concentrated and other parts of the country that share the remaining number of working pharmacists.

M) Pharmaceutical human resource development

The current training content and mechanisms for deployment of pharmaceutical personnel have been identified as major constraints to their utilization. Pharmacy training is largely oriented towards clinical practice and general theoretical knowledge, with minimal emphasis on other skills like procurement and supply, manufacturing and trade, etc. that are required to handle the wide pharmaceutical sector functions.

There is need to ensure their continuous training and capacity building in different pharmaceutical domains including QC and QA.

I.3 Description of the project – Analytical strategies against poor quality medicines

The analytical strategies against poor quality medicines takes into account the lifecycle of method as shown in **Figure 7**.

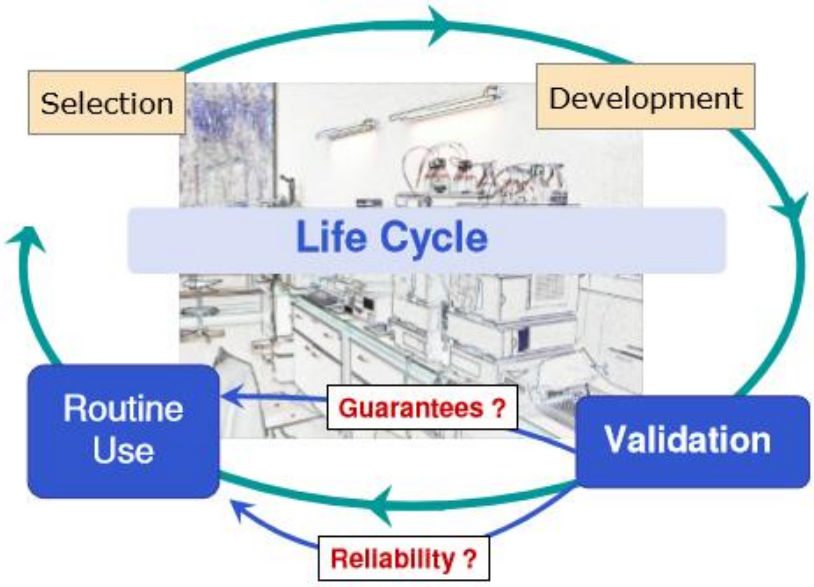


Figure 7 *Analytical method lifecycle*

The first step in this study included the "Method selection" based on the existing analytical techniques and according to the need. However, in the frame of poor-quality medicines (PQM), the challenge is that the real composition in active and inactive ingredients is never known both qualitatively and quantitatively.

The liquid chromatography (LC) technique coupled with UV detector or DAD is usually considered as reference analytical technique for chemical separation, quantification and identification of compounds in samples. It is very used in pharmaceutical analysis due to its sensitivity and accuracy, and gives results in relatively reasonable time, generally between 10 and 60 minutes depending on the method. Another advantage is its great capacity of automation [30-31]. Therefore, the LC is a technique of choice in the frame of detecting PQM.

Different laboratories in Rwanda such as the LADAMET, RBC/MPPD, RBC/NRL, RSB, NQCL, etc. have recently acquired HPLC systems and the new methods shall be transferred to those laboratories.

In the second step, "Methods development" needs an approach that focuses on well-known active ingredient(s) and excipients of pharmaceutical classes. Generally, in the frame of detecting PQM, the most appropriate approach is to develop generic analytical methods for simultaneous analysis of several compounds that can be found in fixed-dose combinations thanks to design of experiments (DoE) and design space (DS) methodology. The DoE-DS methodology investigates the impact of chromatographic parameters variations and their interactions on the final separation, and determines the optimal analytical conditions and related ranges of robustness within the studied experimental domain [32-33]. This is important as the purpose of analytical methods is for routine analysis of various medicines at a large scale either in their original forms as brand products or in generic forms. However, in the frame of PQM, method development is challenged by the lack of information on the true composition of the products. The DoE-DS methodology presents another big advantage of creating a useful database during the realization the experimental matrix on different chromatographic conditions; this database can be exploited latter in predicting optimal analytical conditions for the analysis of any further combination of compounds involved in the previous DoE-DS based method development. Moreover, the development of methods can be done by "Adjustments or adaptation" of other existing methods without passing through several experimental tests of a full DoE, but simply by fitting the methods to the formulations that have almost same composition as that of the products analyzed with the first methods.

However, although there is HPLC equipment in Rwanda, the development of new methods is not widely known or applied.

In the third step, "Method validation" is a requirement to guarantee the reliability of future analytical results. The validation procedure applies to newly developed methods or to the modified/adapted methods from preexisting ones such as compendial monographs and other earlier developed methods that have been amended.

In the pharmaceutical analysis framework, the recommendations on analytical methods validation are based on the guidelines of the International Conference on Harmonization of

Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH). The document *"Validation of Analytical Procedures: Text and Methodology (ICH-Q2R1)"* is the core document on analytical methods validation [34].

The validation process which should demonstrate that an analytical procedure is suitable for its intended purposes consists of a series of performance characteristics shown in **Table 3** depending on the type of analysis to be performed.

Table 3 *Typical analytical methods validation characteristics*

Type of Analytical Procedure:	**Identification**	**Testing for impurities**		**Assay:**
Characteristics		*Quantitative*	*Limit*	*- Dissolution* *- Content/potency*
Accuracy	-	+	-	+
Precision				
Repeatability	-	+	-	+
Intermediate precision	-	+ (1)	-	+ (1)
Specificity (2)	+	+	+	+
Detection limit	-	- (3)	+	-
Quantitation limit	-	+	-	-
Linearity	-	+	-	+
Range	-	+	-	+

Legend:
- : characteristic not normally evaluated
+: characteristic normally evaluated
(1): reproducibility (i.e. inter-laboratory assessment) performed, intermediate precision is not needed
(2): specificity of one analytical procedure compensated by other supporting analytical procedure(s)
(3): may be needed in some cases (e.g.: analysis of subdegradation products, impurity profiles, etc.)

Together with the ICH guidelines, there is another innovative methodological approach developed by the SFSTP (French Society of Pharmaceutical Science and Technology) Commission. These guidelines apply the concept of the total error strategy (i.e. systematic and random errors) expressed as *"bias + standard deviation"* or *"trueness + precision"* illustrated by accuracy profiles as decision tool on the fitness of the methods for their intended use [35].

In Rwanda the QC laboratories use conventional methods such as pharmacopoeias, which once applied on unusual products such as PQM become limited in terms of results reliability due to lack of suitability on the new types of samples. Then, other analytical methods can be applied in the analysis of non-monographed products, or of PQM suspected to contain unusual compounds (APIs and/or excipients) that need to be elucidated. Thus, in the absence of appropriate internal methods, those laboratories can use other methods developed and validated elsewhere by transferring them into their procedures.

Transfer of analytical procedures to host laboratories for routine analysis:

When a new method is validated in the developer laboratory that is skilled and accredited, it can be transferred to the receiver laboratories for routine analyses. In fact, method transfer is a fundamental step in ensuring that the analytical results that will be generated by the receiver (or host) laboratories will be of the same quality as those provided by the developer laboratory.

Another advantage of analytical methods transfer is to qualify the receiving laboratory(s) for using the analytical procedures that are originated from the developer laboratories, thus ensuring that the receiving laboratories have the procedural knowledge and ability to perform the transferred analytical procedures as intended [36-37]. Then, the transfer of analytical procedure must be properly documented according to the quality assurance requirements, and ensuring the reproducibility.

The United States pharmacopoeia (USP) - General Chapter <1224> describes different types of analytical methods transfer:

- **Comparative testing** that requires the analysis of a predetermined number of samples of the homogeneous and same lot by both the sending and the receiving units.

- **Co-validation** between laboratories that is a validation process involving the receiving unit as part of the validation team, and thereby its data is being part of the whole validation data as the assessment of reproducibility.

- **Revalidation** is a partial validation of the analytical procedures by the receiving unit, in which the validation characteristics which are anticipated to be affected by the transfer should be addressed.

- **Transfer waiver** that is an appropriately justified omission of the transfer process under certain circumstances such as :

  - The new product's composition is comparable to that of an existing product and/or the concentration of active ingredient is similar to that of an existing product and is analyzed by procedures with which the receiving unit already has sufficient experience.
  - The analytical procedure being transferred is described in the standardized compendial monographs and is unchanged.
  - The analytical procedure transferred is the same as or very similar to a procedure already in use.
  - The personnel in charge of the method development, validation, or routine analysis at the transferring unit are moved to the receiving unit.

If eligible for transfer waiver, the receiving unit should document it with appropriate justifications.

By considering these different types of transfer of analytical procedures, all of them are applicable in Rwanda, but they need to be well understood and introduced in different laboratories for efficient use whenever needed. So far, comparative testing is applied in the frame of East African Community State members under EAC – Pharmaceutical Proficiency Testing (PT) project organized and sponsored by PTB (National Metrology Institute of Germany) and EAC Secretariat. The Rwandan laboratories participating in the EAC Proficiency Testing are the RBC/MPPD, RSB/NQCL, and the UR/LADAMET.

Application of the method in routine is the step that is quite critical since a laboratory has to demonstrate that the results obtained are not a fact of chance but deserved authenticity. On the other hand, the clients receive laboratory results (as certificate of analysis) without giving details on statistical calculations and reliability (validation) of applied analytical methods. The

requirement is not easy to fulfill since in routine, some variations may appear in pharmaceutical composition (qualitative and quantitative) either without or with intention i.e. unknown compounds to detect in PQM. Therefore efficient analytical techniques in the identification of unknown components in counterfeit medicines have to be used such as [38-39]:

- **Raman spectroscopy:** It is a vibrational spectroscopy technique based on the inelastic scattering of a monochromatic light when interacting with the material to be analyzed. Raman spectra provide qualitative and quantitative information on the active pharmaceutical ingredients (APIs) as well as on the composition of the sample matrices (excipients) that can be present in a variety of pharmaceutical formulations. Moreover, this technique has advantages of being non-destructive, fast, requiring little or no sample preparation, and it is environmental friendly [40-42].

- **Mass spectrometry:** Mass spectrometry measures the mass-to-charge ratio (m/z) of molecules and molecular fragments. The sample is vaporized and then ionized and finally its constituents are separated by an electromagnetic field before reaching a detector. This qualitative and quantitative technique allows the mass of individual molecules or fragments to be determined quickly and accurately [30; 39].

  Coupled with various front-end sample preparation techniques, MS can provide unambiguous fingerprints of complex pharmaceutical samples and therefore enabling counterfeit products being readily identified. Especially useful in this regard are techniques such as DART (direct analysis in real time), which allow much easier MS analysis without extensive sample preparation. Mass spectrometry can be used as a standalone technique or can be coupled with a preceding sample separation stage such as gas chromatography, liquid chromatography, or capillary electrophoresis for better analytical results in particular cases.

- **Nuclear magnetic resonance spectroscopy** that is most known as **NMR spectroscopy** was selected as very powerful analytical technique for the characterization of the structure of drug related substances presented as raw materials or finished products.

This technique excels in the identification of unknown compounds such as metabolites or drug degradation products. It is also used for impurity profiling or determination of the drug's optical purity, and increasingly has a crucial role to play in fighting against poor quality medicines in general by enabling advanced analysis (qualitatively and quantitatively) of unknown compounds in their formulations [30; 43-46].

The targeted applications of the NMR technique in pharmaceutical analysis are mostly based on the following [46]:

- Structure elucidation
- Chemical composition determination
- Formulations investigation
- Raw materials fingerprinting
- Mixture analysis
- Sample purity determination
- Quality assurance and quality control
- Quantitative analysis
- Compound identification and confirmation
- Analysis of inter- and intramolecular exchange processes
- Molecular characterisation
- Reaction kinetics examination
- Reaction mechanism investigation

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PART II - OBJECTIVES

II.1 General objective

The main objective of this thesis is to contribute to the protection of public health in Rwanda by means of setting up efficient generic analytical methods using liquid chromatography (LC) that have to be robust, fast and affordable to fight against poor quality medicines.

II.2 Specific objectives

This general objective is subdivided into four specific objectives that would lead to the achievement of the overall goal of the present thesis.

(i) Development of efficient and reliable generic analytical methods for the screening of antimalarial and Antiretroviral medicines used in Rwanda, or specific methods for the analysis of alone or combined active ingredients;

(ii) Validation of those new analytical methods using the total error strategy combined with accuracy profiles as decision tool to fit for purpose;

(iii) Transfer of those methods from the University of Liège (Belgium) to Rwanda for their further use in routine analysis;

(iv) Utilization of those new methods in routine for analysis of antimalarial and ARV medicines especially when detecting counterfeits, but also in regular quality control of those medicines throughout the distribution chain. That is, use on samples taken before shipment, at the time of delivery and after distribution to ensure that the considered medicines meet quality standards.

PART III - MATERIALS AND METHODOLOGY

III.1 Generality on Materials and Methodology

Liquid chromatography (LC) coupled with simple UV detector or diode array detector (DAD) was selected as conventional analytical technique for simultaneous analysis of several compounds in one sample. This technique is suitable in detecting unknown or known compounds in counterfeit/falsified, substandard and degraded samples.

The selected mobile phases are classical composed with affordable solvents and chemicals which are easily to procure. They are composed of methanol and volatile buffer solutions mainly prepared with ammonium formate salt, but also with ammonium acetate and ammonium dihydrogenophosphate when needed. The advantage of choosing volatile buffering solutions is that they do not precipitate in the mobile phase flow as can do other salts if not very well prepared, and this can obstruate the system causing overpressures, and stopping the system until maintained or repaired. Another reason is the transfer of the method to mass spectrometry (MS) detection for further investigations.

The selected analytical columns are octyl (C8) and octadecyl (C18) silica based stationary phases that are very classical too and widely used in the analysis of organic compounds with pharmacopoeia monographs or other officially approved compendia. Then, both gradient and isocratic elution of mobile phases were used depending on the type/nature and number of analytes in the samples. Gradient mode was selected to separate compounds that have almost similar chromatographic behaviors and isocratic mode for easily separable compounds as per their physicochemical properties and number of expected analytes in the samples. Logically, there is no need to develop gradient methods for the separation of one or two analytes unless other scientific reasons are exist.

The methods with gradient mode were developed considering the DoE-DS approach while those based on simple isocratic mode not requiring long experimental runs were developed by adaptation or adjustment of other existing methods on similar or comparable analytes.

On the other hand, other analytical techniques such as Raman spectroscopy, Mass spectrometry and NMR spectroscopy were found useful to complete or to confirm HPLC results especially for advanced analysis of detected counterfeit medicines in order to elucidate

their real composition, identify any unusual excipients or unknown compounds and therefore prevent the users from any dangers.

In our study, both geometric and geographic transfers were planned in order to reduce the global cost analysis. Geometric transfer is related to methods transfer from long to short analytical columns that will allow to reduce the analysis time and solvents consumption, whereas geographic transfer is about transferring the methods from the developer laboratory in Belgium to Rwanda that will allow the analysis locally; noted that time is very important in the detection of PQM as soon as possible in order to allow health systems using quality checked medicines and avoid using inadequate or harmful ones.

III.2 Methodology on sampling countrywide

Our sampling strategy is based on targeting suspected or risky places where PQM can be found. They can be towns, suburbs, and country borders for both public and private drug distribution points mainly pharmacies and hospitals. Any possible informal channels of medicines are also targeted. Then, the targeted medicines were antimalarial and antiretroviral medicines officially used in Rwanda.

As illustrated in **Figure 8**, sampling sites include:

- Areas with intensive pharmaceutical commercial activities,
- Border areas with other countries (Burundi in South, Tanzania in East, Uganda in North, and D.R. Congo in West) where the risk of illegal trade of goods including medicines from one country to another is relatively high and therefore the possibility of poor quality medicines especially counterfeit/falsified ones in circulation.

All samples were randomly selected by considering batch numbers, manufacturers, strength, and dosage forms (tablets, capsules, injectables, suspensions, suppositories, etc.).

On the other hand, there are other samples that were received from partners networking in Kinshasa (D.R. of Congo), in Cotonou (Benin) and locally (Rwanda) in the framework of scientific collaboration between those partnerships that includes the University of Liège

(Belgium), the University of Kinshasa (DRC) and the University of Abomey Calavi (Benin) as well as the University of Rwanda. Samples of antimalarial medicines were concerned since malaria is endemic in these countries generally having the same suppliers of generic or brand medicines, and therefore the same type counterfeiting could be found.

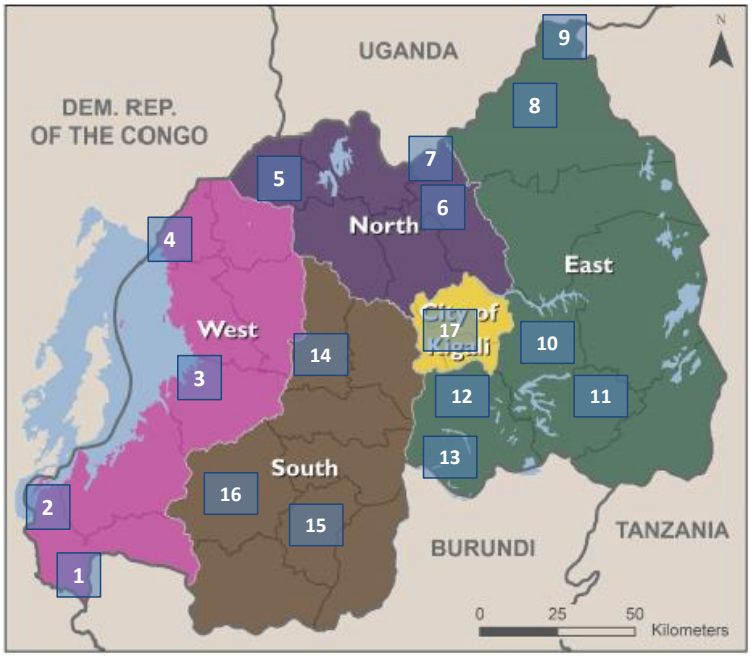

Figure 8 *Strategic sampling sites on the map of Rwanda (**Western Province:** (1) Bugarama, (2) Kamembe, (3) Karongi/Kibuye, (4) Rubavu/Gisenyi; **Northern Province:** (5) Musanze/Ruhengeri, (6) Gicumbi/Byumba, (7) Gatuna; **Eastern Province:** (8) Nyagatare, (9) Kagitumba, (10) Rwamagana, (11) Ngoma/Kibungo, (12) Nyamata/Bugesera, (13) Ruhuha/Ngenda; **Southern Province:** (14) Muhanga/Gitarama, (15) Huye/Butare, (16) Nyamagabe/Gikongoro; and **Kigali City:** (17) The Capital).*

The sampling for ARV medicines was done at the RBC/MPPD (national drug store based at Kigali the capital of Rwanda), CHUB (Butare teaching university hospital in Southern Province), and Nyamasheke Pharmacy District in the Western Province. We selected few sampling places for ARVs unlike for antimalarial medicines because they are mostly dispensed in public health institutions, and are strictly regulated by the Ministry of Health and the international partner donors who procure them from WHO prequalified manufacturers.

PART IV - RESULTS

IV.1 SECTION 1: Application of Design Space Optimization strategy to the Development of LC methods for simultaneous analysis of 18 antiretroviral medicines and 4 major excipients used in various pharmaceutical formulations

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IV.1.1 Preamble to Article 1

The last Joint United Nations Programme report on HIV and AIDS (UNAIDS, 2016) on Rwanda presented alarming figures as following [1]:

- The number of adults and children living with HIV was around 220 000 [210 000 – 250 000], the figures in […] represent lower and upper estimates;
- The prevalence rate of HIV among adults aged 15 to 49 years was 3.1% [2.6 % – 3.5%];
- The people living with HIV who knew their status were 200 000;
- The people who were on antiretroviral therapy (ART) 180 000; and
- Death to adults and children due to AIDS was approx. 3 300 [2 500 – 4 200**]**.

Antiretroviral medicines are new molecules in relation with **Figure 8** (p.12) that classified HIV/AIDS among the top 10 causes of death and disability combined (DALYs) in Rwanda. The country is also benefiting from new ARV molecules that are under constant pharmaceutical development and innovation to make them more effective. Beside this effort, there is need of having suitable analytical methods to support the Government's initiatives and international organizations engaged in fighting against that terrible disease, such as the Global Fund, UNAIDS (Joint United Nations Programme on HIV/AIDS), PEPFAR (United States President's Emergency Plan for AIDS Relief), Bill & Melinda Gates Foundation, etc. through reliable quality control of pharmaceutical products at different stages of the supply chain (pre-shipment, at delivery, and post-market inspections), in routine analysis, and particularly in counterfeiting detection.

[1] Source: UNAIDS | AIDSinfo, Country factsheets RWANDA | 2016. Retrieved May 23, 2018 from http://www.unaids.org/en/regionscountries/countries/rwanda

In this context, the first part of our results tries to answer to the needs that become a challenge towards therapeutic changes due to the existence of different fixed-dose combinations in several active ingredients, substitution of those APIs by others that are more effective, and modification of dosage forms for adults and pediatrics. In anticipation of this challenge, a design of experiments and design space methodology (DoE-DS) was necessary for the development of HPLC methods for simultaneous analysis of 15 antiretroviral active ingredients and 4 major excipients that we selected.

At first, the experiments were planned on the basis of D-optimal experimental design conducted separately on four subgroups of the selected analytes. This step is critical due to potential errors that may occur while collecting chromatographic data that may be biased by peak overlapping. To avoid this situation, the analytes were regrouped by considering their pKa(s) and molecular weights. Then, predictions of retention times (t_R) of all peaks were made for a mixture solution containing the selected molecules, allowing to define a design space where the separation criterion (S) was ≥ 0 min. Three Design Spaces were defined: one for the screening of all the 15 ARVs; one for the analysis of liquid dosage forms containing 4 preservatives; and one for solid dosage forms.

After methods optimization, one fixed-dose combination composed by emtricitabine (FTC), tenofovir disoproxil fumarate (TDF) and efavirenz (EFV) was fully validated according to the ICH Q2(R1) guidelines and Total Error strategy using the accuracy profiles as decision tool. The same method was tested to 3 other ARV active ingredients which were initially not considered, as being introduced latter in 2015 in the national list of essential medicines by the Government of Rwanda, while our experiments were started in 2014.

IV.1.2 Article 1

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Application of design space optimization strategy to the development of LC methods for simultaneous analysis of 18 antiretroviral medicines and 4 major excipients used in various pharmaceutical formulations

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ABSTRACT

As one of the world's most significant public health challenges in low- and middle-income countries, HIV/AIDS deserves to be treated with appropriate medicines, however which are not spared from counterfeiting. For that, we developed screening and specific HPLC methods that can analyze 18 antiretroviral medicines (ARV) and 4 major excipients. Design of experiments and design space methodology were initially applied for 15 ARV and the 4 excipients with prediction thanks to Monte Carlo simulations and focusing on rapidity and affordability thus using short column and low cost organic solvent (methanol) in gradient mode with 10 mM buffer solutions of ammonium hydrogen carbonate. Two other specific methods dedicated to ARV in liquid and in solid dosage formulations were also predicted and optimized. We checked the ability of one method for the analysis of a fixed-dose combination composed by emtricitabine/tenofovir/efavirenz in tablet formulations. Satisfying validation results were obtained by applying the total error approach taking into account the accuracy profile as decision tool. Then, the validated method was applied to test two samples coded A and B, and claimed to contain the tested ARV. Assay results were satisfying only for sample B.

1. INTRODUCTION

HIV/AIDS remains one of the world's most significant public health challenges. The World Health Organization (WHO) reports that at the end of 2015, there were approximately 36.7 million people living with HIV, with 2.1 million people becoming newly infected in the same year globally. Low- and middle-income countries and particularly in Sub-Saharan Africa are the most affected with 25.6 million people (i.e. around 70% worldwide) living with HIV [1, 2].

On the other hand, antiretroviral (ARVs) medicines are not spared from counterfeiting, especially for their substantial high unit costs, long term medical treatment and sustained demand. For example, in 2003 the WHO issued an alert that a product called 'Ginovir 3D', marketed in Ivory Coast as a combination of triple ARV was counterfeit containing only one of the active ingredients and another non-declared ARV agent [3]. In 2004 “Médecins Sans Frontières” discovered counterfeit ARVs on the market in the Democratic Republic of Congo that contained an antidepressant and a muscle relaxant [4]. In 2011 the Government of Kenya removed thousands of batches of ARVs from circulation after patients and health workers reported irregularities in the appearance and texture of a widely used antiretroviral Zidolam - N [5, 6].

Furthermore, as the antiretroviral therapy (ART) constitutes a growing pharmaceutical research field that necessitates combining several ARV molecules to maximally suppress HIV and stop the progression of HIV disease, there is need of disposing rapid and efficient analytical methods that can serve in different stages of drug development lifecycle, to ensure quality and guarantee safety and efficacy. There are also few monographs dedicated to the analysis of multiple ARV combinations already on the market, for example there is no monograph yet for the analysis of efavirenz/lamivudine/tenofovir in the three most popular pharmacopoeias i.e. International Pharmacopoeia (Ph. Int.), British Pharmacopoeia (BP) and United States Pharmacopoeia and the National Formulary (USP-NF).

In the frame of fighting against poor quality antiretroviral medicines especially counterfeit/falsified ones, we aimed to develop effective, rapid and affordable methods. In this context HPLC methods were preferred considering classical and short analytical columns and low cost mobile phase such as methanol with classic buffer solutions. Three types of methods

were targeted depending on the situation that will be faced : (*i*) the screening methods for which the interest will be the analysis of suspected counterfeit ARV medicines or complex samples that contain several ARV molecules, (*ii*) the generic method(s) that are expected for the analysis of specific cases for example of different fixed-dose combinations (FDC) in liquid dosage forms containing generally preservatives and/or anti-oxidants, and (*iii*) the generic method(s) applicable to different FDCs in solid dosage forms not generally containing preservatives or anti-oxidants.

This study focused on 18 antiretroviral medicines widely used in HIV/AIDS treatment, and 4 major preservatives and anti-oxidants generally used in liquid dosage formulations. Their chemical structures are given in **Fig. 1**.

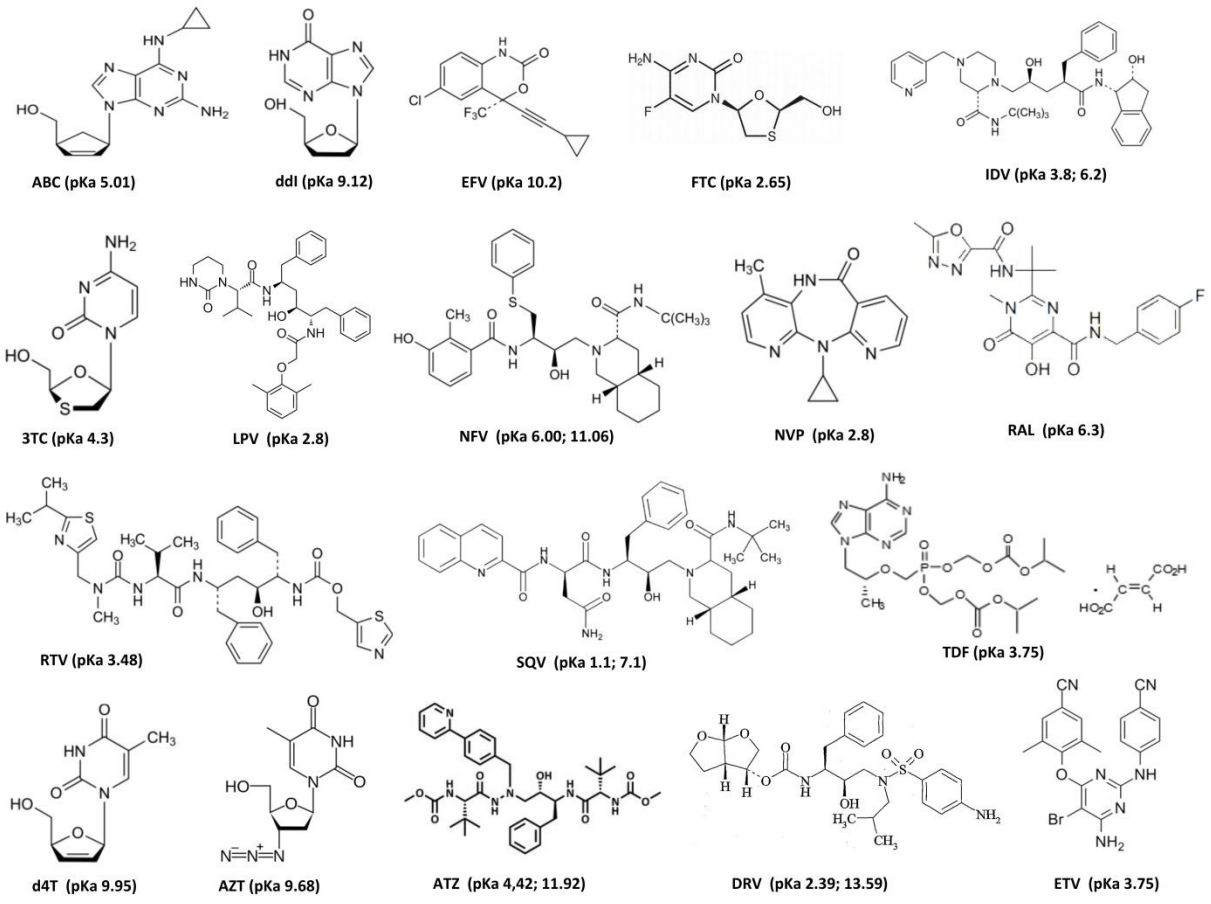

Fig. 1 *Molecular structures and pKa(s) of the 18 antiretroviral medicines*

Legend:
ABC: Abacavir, ATZ : atazanavir, ddI : didanosine, DRV : darunavir, d4T : stavudine, EFV : efavirenz, ETV : etravirine, FTC : emtricitabine, IDV : indinavir, LPV : lopinavir, NFV : nelfinavir,

NVP : nevirapine, RAL : raltegravir, RTV : ritonavir, SQV : saquinavir, TDF : tenofovir, ZDV or AZT : zidovudine, 3TC : lamivudine

A strategic approach based on design of experiments (DoE) and design space (DS) methodology was followed during the development of methods. It presents advantages of allowing the exploration of different chromatographic factors effects and the interactions between them. On the other hand, DS defined as "multidimensional combination and interaction of input variables that have been demonstrated to provide assurance of quality" is suitable to predict optimal analytical conditions within the experimental domain [7-10]. Then in order to ensure its fit-for-purpose, one of the candidate methods was submitted to validation prior to its use in routine analysis and its proposal to sustain monographs. We privileged the concept of total error strategy using accuracy profiles as decision tool while considering the ICH Q2 (R1) guidelines about the validation of analytical procedures [11-15].

2. EXPERIMENTATION

2.1. Materials and Methods

2.1.1. Chemicals

Abacavir sulfate (99.4%), atazanavir sulfate (99.6%), nelfinavir mesylate (98.3%), ritonavir (99.4%), saquinavir mesylate (99.4%), tenofovir disoproxil fumarate (99.1%), and zidovudine (99.0%) were purchased from the United States Pharmacopeial Convention (Rockville, USA), darunavir ethanolate (100.0%), didanosine (> 99%), efavirenz (> 99%), emtricitabine (> 99%), etravirine (97.1%), lopinavir (> 99%), nevirapine (> 99%) and raltegravir potassium salt (> 99%) from Alsachim (Strasbourg, France), indinavir (97.2%), lamivudine (99.7) and stavudine (99.6%) from the European Directorate for the Quality of Medicines "EDQM" (Strasbourg, France). The four preservatives used in this study were obtained from the following suppliers: butylated hydroxyanisol "BHA" (96%) and methyl paraben "nipagin" (99%) from Alfa Aesar GmbH & Co KG (Karlsruhe, Germany), butylated hydroxytoluene "BHT" (≥ 99%) from Merck Schuchardt OHG (Hohenbrun, Germany), and propyl paraben "nipasol" (101.9%) from Fagron N.V. (Waregem, Belgium). Ammonium formate (99%) was purchased from Alfa Aesar (Karlsruhe, Germany), methanol (HPLC gradient grade) and

hydrochloric acid (37%) from Merck (Darmstadt, Germany). The ultrapure water was produced with a Milli-Q Plus 185 water purification system (Millipore, Billerica, MA, USA).

2.2. Sample preparation

2.2.1. Sample solutions for methods development

Three types of solution were concerned:

2.2.1.1 Individual sample solutions for preliminary data information

Fifteen individual sample solutions at 100 µg mL^{-1} of ABC, ddI, EFV, FTC, IDV, 3TC, LPV, NFV, NVP, RAL, RTV, SQV, TDF, d4T, and AZT were prepared in methanol by dissolving approximately 10.0 mg of each individual analyte in 100.0 mL volumetric flasks, and completing to volume with the same solvent. For non-easily soluble compounds in methanol, 10 minutes of ultrasonic bath were necessary to allow total dissolution.

These solutions served in collecting preliminary data information on respective UV spectra and retention times at apex (t_R). The individual sample solutions for BHA, BHT, NpG and NpS were not prepared as the needed preliminary information was already known and available. Collecting the retention time of the single compounds prior to optimization was useful in order to divide the compounds into subgroups and avoid peak overlapping that would be observed when testing all the compounds together. Indeed, in order to properly compute a DS, all peaks should be fully monitored for the whole range of experimental conditions.

2.2.1.2 Sub-group solutions for design of experiments (DoE)

The 15 ARV compounds and the 4 excipients were split into four sub-groups differing from one to another by respective molecular weights and dissociation constants “pKa(s)” as shown in **Table 1**. We tried as much as possible to have sub-groups containing molecules with different analytical data to easy the experimental design.

Stock solutions per sub-group were prepared at 1 mg mL^{-1} of each analyte in methanol, and 10 minutes of ultrasonic bath were necessary to allow total dissolution of non-easily soluble compounds. Then, aliquots from sub-group stock solutions were diluted with the same solvent to have final working solutions at 200 µg mL^{-1} for design of experiments.

Table 1

Grouping of analytes per pKa(s) and molecular weights in different sub-group solutions for DoE

Sub-group	Analytes	pKa(s)	Molecular weight (g/mol)
Sub-group 1	ABC	5.01	335.37
	BHA	8.9	180.25
	ddI	9.12	236.23
	d4T	9.95	224.21
	EFV	10.2	315.67
Sub-group 2	NVP	2.8	266.30
	IDV	3.8; 6.2	711.87
	RAL	6.3	444.42
	AZT	9.68	267.24
	BHT	12.23	220.36
Sub-group 3	FTC	2.65	247.25
	TDF	3.75	635.51
	3TC	4.3	229.26
	NFV	6.00; 11.06	663.90
	NpG	8.4	152.15
Sub-group 4	SQV	1.1; 7.1	670.84
	LPV	2.8	628.81
	RTV	3.48	720.94
	NpS	7.91	180.20

2.2.1.3 Overall solution for the screening method

A mixture of aliquots was prepared by mixing together 2.0 mL from each sub-group stock solution in a 10.0 mL volumetric flask in order to have a final solution of 200 µg mL^{-1} of each analyte. This overall working solution was used for testing the optimal analytical conditions of the screening method and also for assessing other predicted methods to check their capability in separating the analytes in the overall mixture solution.

2.2.1.4 Additional sub-group of other ARV compounds after design of experiments

Another sub-group i.e. Sub-group 5 composed by three additional ARV agents namely ATZ (pKa 4.42; 11.92), DAR (pKa 2.39; 13.59) and ETV (pKa 3.75) was prepared in the same conditions as done for the four previous sub-groups in order to have final working solutions at 200 µg mL^{-1} (see section ii). In fact, the present study including the planed DoE was initially based on 15 ARV medicines used in Rwanda by 2013 and inventoried from the national list of essential medicines published at that period [16]; After having constructed the DoE, we were informed that in 2015 the Government of Rwanda that served us as a model of developing country using ARV medicines at a large scale has updated its national list of essential medicines by introducing the three ARV above mentioned in the ART for adult and pediatric patients [17-18]. This new sub-group was used to check whether the analytical method for the analysis of solid dosage forms will be suitable to detect the three compounds which are among the most used in pharmaceutical solid dosage forms.

2.2.2. Sample solutions for calibration and validation

Two sets of solutions, one for calibration standards (CS) and another for validation standards (VS) were prepared, by dissolving in methanol approximately 7.0 mg of FTC, 10.0 mg of TDF, and 20.0 mg of EFV in each volumetric flask of 20.0 mL and completed to volume with the same solvent to obtain stock solutions at the concentration of 350 µg mL^{-1}, 500 µg mL^{-1} and 1000 µg mL^{-1} of FTC, TDF and EFV, respectively.

Then, appropriate and subsequent dilutions were performed using methanol in order to obtain three final calibration standards (CS) at three different concentration levels, and three validation standards (VS) at five concentration levels daily.

A) Calibration standards

The corresponding concentration levels for CS were as following:

Level 1 (50%): 42 µg mL^{-1} (FTC), 60 µg mL^{-1} (TDF) and 120 µg mL^{-1} (EFV);
Level 3 (100%): 84 µg mL^{-1} (FTC), 120 µg mL^{-1} (TDF) and 240 µg mL^{-1} (EFV);
Level 5 (150%): 126 µg mL^{-1} (FTC), 180 µg mL^{-1} (TDF) and 360 µg mL^{-1} (EFV).

B) Validation standards

The validation standards (VS) were prepared considering the tablets matrix in order to simulate as much as possible the drug formulation and evaluate the matrix effect on the analytes. A matrix stock solution composed by major excipients of the studied medicine was prepared by dissolving titanium dioxide, talc, croscarmellose sodium, magnesium stearate, microcrystalline cellulose, sodium laurylsulfate and hyprolose in methanol in order to obtain 0,1 mg mL^{-1}, 0,1 mg mL^{-1}, 5 mg mL^{-1}, 0,5 mg mL^{-1}, 25 mg mL^{-1}, 1 mg mL^{-1} and 0,5 mg mL^{-1} respectively. This solution contains an equivalent amount of the major excipients as compared to the real galenic formulation for which the analytical method is validated for.

For VS, one stock solution containing FTC at 350 µg mL^{-1}, TDF at 500 µg mL^{-1} and EFV at 1000 µg mL^{-1} was prepared in methanol as done for CS. To this stock solution was added 545 µL of the matrix stock solution in a volumetric 20.0 mL flask and completed to volume with methanol. This solution was filtered through 0.45 µm PTFE syringe filtration disks before diluting the aliquots for experiments.

Appropriate dilutions were performed with methanol in order to obtain final validation standard solutions at five different concentration levels, and three independent solutions per concentration level:

Level 1 (50%): 42 µg mL^{-1} (FTC) - 60 µg mL^{-1} (TDF) and 120 µg mL^{-1} (EFV);
Level 2 (75%): 63 µg mL^{-1} (FTC) - 90 µg mL^{-1} (TDF) and 180 µg mL^{-1} (EFV);
Level 3 (100%): 84 µg mL^{-1} (FTC) - 120 µg mL^{-1} (TDF) and 240 µg mL^{-1} (EFV);
Level 4 (125%): 105 µg mL^{-1} (FTC) - 150 µg mL^{-1} (TDF) and 300 µg mL^{-1} (EFV);
Level 5 (150%): 126 µg mL^{-1} (FTC) - 180 µg mL^{-1} (TDF) and 360 µg mL^{-1} (EFV).

In final, three independent solutions (n = 3) were prepared for each concentration level (m = 3 for CS, m = 5 for VS), and all these preparations were repeated for three days corresponding to three series (p = 3).

For the analyses of medicine samples, the concentrations of reference standard solutions were identical to Level 3 (100%) of the VS: 84 µg mL^{-1} of FTC, 120 µg mL^{-1} of TDF and

240 µg mL^{-1} for EFV. For the sample to be analyzed, powdered portions of the tablets were taken and treated in the same way as reference solutions to give theoretically final expected concentrations of 84 µg mL^{-1} (FTC), 120 µg mL^{-1} (TDF) and 240 µg mL^{-1} (EFV). The solutions were freshly prepared, and they were filtered through 0.45 µm PTFE syringe filtration disks prior to their analysis onto the liquid chromatographic system.

2.2.3 Sample preparation for FTC/TDF/EFV tablets

20 tablets from each sample batch were weighed and finely powdered. Then, three portions containing approximately 80.0 mg of FTC, 120.0 mg of TDF, and 240.0 mg of EFV for the sample containing FTC/TDF/EFV were accurately weighed and dissolved in 100.0 mL volumetric flasks with methanol. One minute of mechanical shaking with vortex was enough to dissolve the studied analytes; and further appropriate dilutions were done with the same solvent to have final working solutions containing 80/120/240 µg mL^{-1} of the three active ingredients respectively in sample A, and 80/120 µg mL^{-1} in sample B containing only FTC and TDF. But before injecting the sample solutions in the chromatographic system, they were filtered through 0.45 µm PTFE syringe filtration disks.

In parallel, two independent standard solutions were prepared at the same concentration levels (i.e. 80/120/240 µg mL^{-1}) of FTC, TDF, and EFV reference substances prepared in methanol too.

2.3. Instrumentation and chromatographic conditions

The optimization of the methods was performed on a HPLC system comprising a Waters 2695 separation module coupled to a Waters 2996 photodiode array detector from Waters Corporation (Eschborn, Germany). The analytical column was XBridge Shield (100 mm × 4.6 mm ID), packed with C18 stationary phase (3.5µm, dp) from Waters Corporation.

The analytes were monitored photometrically at a wavelength of 210 nm while chromatographic data of experimental conditions were recorded from 200 to 400 nm. The collected chromatographic data were basically the UV spectra of the analytes, retention times (at the beginning, apex and end), peaks' areas and peaks' heights. The injection volume was 10 µL and the mobile phase flow rate was 1 mL min^{-1}, the temperature of the column

compartment was fixed at values between 25°C and 35°C depending on the DoE value to be tested or optimized method. The temperature of sample compartment was fixed at 15°C.

Measurements of pH were performed with a SevenEasy S20 pH meter (Mettler Toledo, Columbus, OH, USA). The buffer solutions of the mobile phase consisted of ammonium formate with pH adjusted between 2.7 and 4.8, of ammonium acetate with pH adjusted between 4.8 and 6.0, and of ammonium hydrogen carbonate with pH adjusted between pH 6.0 and 10.0. The adjustment depended on the values to test for DoE and for the optimal analytical conditions. The pH was adjusted with 6N hydrochloric acid or with ammonium hydroxide 27% while the concentration of the buffers was 10 mM.

2.4. Software

Empower 2.0 software (Waters Corporation, MA, USA) for Windows was used to control the HPLC system, to record the signals from the detector and to interpret the generated chromatograms. The gradient steps were calculated using a freeware HPLC Calculator v3.0 developed by Guillarme et al. (2008) [19]. JMP 12.2.0 (SAS Institute Inc., NC, USA) and R 3.2.2 (GNU project / Free Software Foundation, MA, USA) softwares were used for generating the DoE trials and experimental data treatment. The accuracy profiles as well as the statistical calculations including the validation results and uncertainty estimates were obtained thanks to e-noval® V3.0 software (Arlenda, Belgium).

3. RESULTS AND DISCUSSIONS

3.1. Design of experiments

The strategy of design of experiments (DoE) was applied to collect data through laboratory tests and to define design spaces (DS) related to different optimal analytical conditions for the screening method or specific methods for the analysis of ARV compounds and the studied excipients in different dosage forms. In this study, a D-Optimal design was used to allow simultaneous optimization of the methods in few experimental runs. Each experiment was performed in duplicate considering the mean of the results.

The pH of the aqueous part of mobile phase, the gradient time (TG) and the column temperature (T^o) were selected as the factors to investigate the appropriate analytical conditions within the selected levels of those factors as shown in **Table 2**. All the factor levels were symmetrical. For pH, the symmetry was between values 2 and 8, with 5 the central level and additional points between that central and the extreme points. Since the majority of the compounds to test are basic another point (pH 10) was added to further investigate the separation of those compounds. Because the chromatographic column was able to support that pH level, it was assessed and treated as an augmented level of the initial DoE. Volatile salts were preferred for potential further transfer to LC-MS in case of investigation of potential "abnormal peak", i.e. in counterfeit medicines. A total of 19 experimental conditions were randomly generated with R 3.2.2 software (see **Table 3**). For each trial run, three retention times (t_R), at the beginning, the apex and the end of each peak analyte were recorded as analytical responses from the chromatogram; Retention time at apex was used for peak identification while retention times at the beginning and at end were used to model the responses and express the separation (S). The sample solutions of each sub-group were analyzed throughout these 19 experimental trials.

Table 2

Factors and corresponding levels selected for the D-Optimal Design

Factors	**Levels**					
pH	2.70	4.53	5.85	6.35	8.18	10.00
Gradient time (TG, min.)	7.25		15.25		23.25	
Temperature (T°, °C)	25.0		30.0		35.0	

Prior to run the experimental trials, preliminary tests were carried out at pH 2.7 and pH 10.0, TG = 7.25 min. and T° = 35°C on individual sample solution of each compound. This allowed recording the information on UV-Vis spectra and t_R of each peak analyte useful for easy identification and information on the chromatographic behavior in terms of elution speed.

To prevent any co-elution of the peak analytes during the experiments, we took the precaution of dispatching the analytes according to their differences on pKa(s) of at least 1 unit pKa and molecular weight of 10 g mol^{-1}. This allowed good chromatographic separation of the compounds and therefore allowed recording precise data on respective three retention times

(beginning, apex and end) of each peak analyte since with co-eluting peaks this would not be possible.

Table 3

Experimental matrix of D-Optimal design for the investigation of analytical factors

Trial	Experimental set up		
	T° (°C)	TG (min)	pH
1	35.0	15.25	4.53
2	30.0	23.25	4.53
3	35.0	7.25	10.00
4	25.0	7.25	10.00
5	35.0	15.25	8.18
6	30.0	23.25	10.00
7	30.0	15.25	2.70
8	25.0	7.25	2.70
9	35.0	23.25	10.00
10	25.0	15.25	10.00
11	35.0	7.25	2.70
12	25.0	23.25	2.70
13	30.0	15.25	6.35
14	25.0	7.25	5.85
15	30.0	15.25	6.35
16	30.0	7.25	8.18
17	30.0	15.25	6.35
18	25.0	23.25	8.18
19	35.0	23.25	2.70

3.2. Influence of the analytical factors on the chromatographic behavior of the analytes

The responses (retention times at beginning, apex and end) from the experimental data were modeled by the following multivariate multiple linear model with quadratic for factors other than pH and interactions between factors as shown by the following equation:

$$Y = \beta_o + \beta_1.pH + \beta_2.pH^2 + \beta_3.pH^3 + \beta_4.T_G + \beta_5.T_G^2 + \beta_6.T° + \beta_7.T°^2 + \beta_8.pH.T_G + \beta_9.pH.\ T° + \beta_{10}.T°.T_G + \beta_{11}.pH.T°.T_G + E$$

$$Y = XB + E$$

With βo, … β11, the model parameters and E the estimated error.

As can be noticed in **Fig. 2**, pH and TG have an important influence on the three retention times of the compounds: Obviously the decrease of the TG significantly decreases the three retention times of the compounds, but often at the expense of peak separation due to peak co-elution. It was also noticed that pH variation of the mobile phase led to significant changes in retention times of some compounds such as 3TC, ABC, NpG, NpS, TDF, RAL, d4T, IDV, SQV, and AZT, but also the variation of pH of the mobile phase can cause the inversion of peaks and therefore change the elution order of peaks (see **Fig. 2.A**). For example the elution order at pH 2.7 will start with 3TC while at pH 10.0 it will start with ddI. Other compounds such as ABC, NpG, RAL, NpS, IDV, etc. will have a change in the elution order upon variation of the pH. Moreover, some compounds are subject of co-elution at some pH levels; for example NVP/NpS at around pH 10.0, AZT/NpG at around pH 9.7, 3TC/ddI and RAL/ABC at around pH 8.5, ddI/3TC/FTC/d4T, NpG/ABC, RAL/NVP, and NpS/TDF at around pH 7.0, ddI/FTC/d4T, NpG/ABC, NpS/TDF, and SQV/NFV/LPV at around pH 5.0, AZT/ABC and NpG/NVP at around pH 2.7. Therefore there is need of carefulness while optimizing the methods involving those compounds in a drug formulation.

Concerning T°, one can noticed that when increasing between 25°C and 35°C, a slight retention times decrease of all peak analytes was observed without any co-elution or change of peaks elution order.

Based on these observations, the ideal would be to run the chromatographic analysis at higher TG for better peaks separation thus their easier identification; however, this becomes a compromise with the analysis time that should be reduced in order to obtain the results as rapid as possible for allowing taking decision on analyzed batch products especially in case of failure (out-of-specifications).

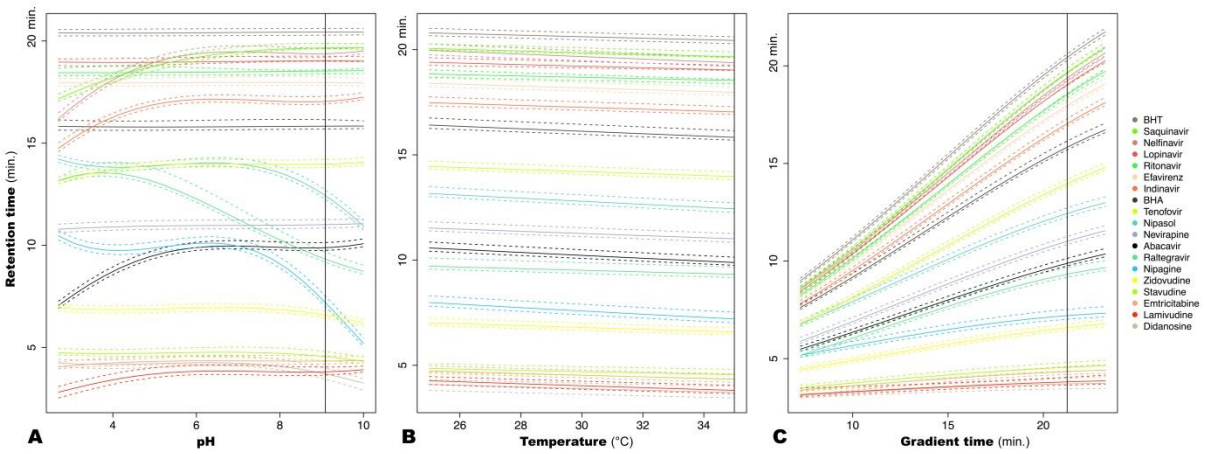

Fig. 2. *Modelled retention times (t_R) of different compounds with respect to factors of experimental design:* ***(a)*** *pH,* ***(b)*** *temperature ($T^{o,}$ ^{o}C), and* ***(c)*** *Gradient time (TG, min)*

Legend:

- BHT
- Saquinavir
- Nelfinavir
- Lopinavir
- Ritonavir
- Efavirenz
- Indinavir
- BHA
- Tenofovir
- Nipasol
- Nevirapine
- Abacavir
- Raltegravir
- Nipagine
- Zidovudine
- Stavudine
- Emtricitabine
- Lamivudine
- Didanosine

Table 4

Optimal analytical conditions and operating range within DS for the separation of the molecules

	Optimal P(S > 0)	**pH**		
Screening method for 18 compounds	0.2%	9.09 (8.70-9.30)	21.25 (21.0-21.6)	35.0 (34.5-35.0)
Method for Liquid dosage forms	95%	3.61 (3.60-6.00)	9.25 (9.0-23.3)	25.0 (25.0-35.0)
Method for Solid dosage forms	95%	2.70 (2.70-3.70)	7.25 (7.0-23.3)	25.0 (25.0-35.0)

3.3. Methods optimization

The optimization of the methods was done thanks to R 3.2.2 freeware [20] available for most computer systems. It allowed to develop Bayesian models on the prior recorded results of the DoE and to predict different optimal analytical conditions for the expected analytical methods

using Monte Carlo simulations. To each expected method, the following were computed: design spaces, graphs of the t_R variation versus T°, pH and TG as well as the corresponding predicted chromatogram. Then, the predicted optimal conditions were tested in practice while maintaining constant the other analytical conditions: column, flow rate, injection volume and detection wavelength as mentioned in the section of materials and methods. As mobile phase, classic and affordable solvents were privileged i.e. methanol LC grade and buffers mentioned earlier (see section 2.3).

Three kinds of optimization were needed in order to obtain the optimal conditions on T°, pH, TG and probability of peaks separation P(S > 0) for the studied analytical methods presented in **Table 4**.

3.3.1 First optimization: Screening method

To sustain the optimization of the screening method through the evaluation of quality chromatogram [7; 8], we selected the separation between peaks of the critical pair as a critical quality attribute (CQA). Then, as proposed by Lebrun et al. [9], we used in this study the separation criterion (*S*) defined as the difference between the t_R at the beginning of a "n" th eluting peak (t_RB) and the t_R of the end of the "n-1" th eluting peak (t_RE) of the critical peak pair. The critical pair is defined by min(S), for a given operating condition. In the present study, it was not constituted by the same peak pairs; it has changed every time within the domain according to the experimental conditions. A screening method able to detecting 15 ARV and 4 preservatives compounds was optimized. The optimized experimental conditions were selected inside the design of experiments by means of the DS and considering the CQA defined as S > 0. These were expressed in terms of probability with the optimal conditions indicated in **Table 4** including the probability surfaces to have S > 0 (see **Fig. 3**). As can be observed over the experimental domain that we investigated, it is noticed that the probability of peak separation P(S > 0) is very low (around 0.2%) the range of the optimal conditions is narrow to allow separation of all peaks, reversely when the probability is very high (95%), the range of the optimal conditions is large (see section 3.3.3.). Such a low probability may signify that a full separation (S > 0) among all the compounds is hardly found. The main reason over this low probability relies in the "too complex" situation depicted hereby indeed; numerous compounds are encompassed in the design space computed. Besides that, another explanation is the chromatographic behavior of compounds such as 3TC, ddI, d4T and FTC

which are similar and that co-elute around 5 minutes (see **Fig. 4.a**). Moreover, this probability is lessened by the calculated uncertainty of prediction represented by the gray zones around all analytes, and therefore allowing to predict a relatively higher risk of having other co-eluting peaks for compounds that have similar chromatographic behavior especially in case of changes of pH and TG as discussed in the previous section about the influence of the studied factors (pH, T°, TG). One can notice potential risk of peak co-elution of AZT and NpG at around 7 minutes; RAL and ABC at around 10 minutes; and RTV, LPV, NFV and SQV at around 19 minutes.

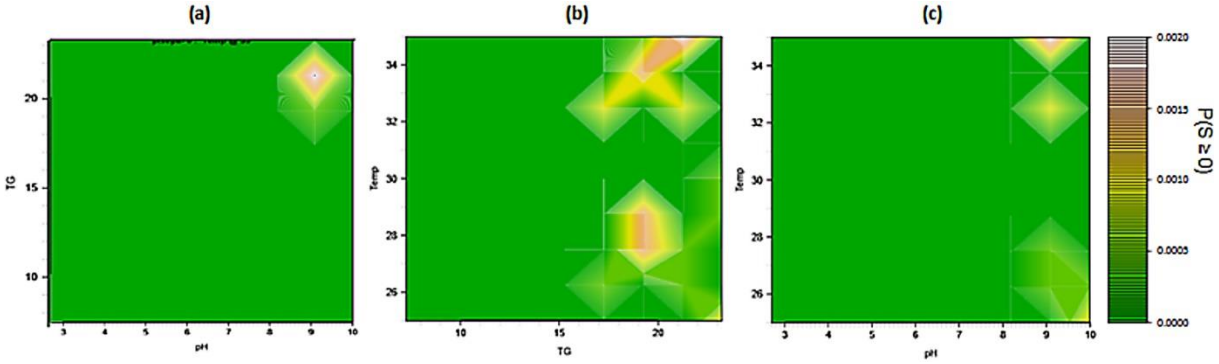

Fig. 3. *Probability surfaces to reach S ≥ 0:* ***(a)*** *pH versus TG (min.),* ***(b)*** *TG (min.) versus T° (°C),* ***(c)*** *pH versus T° (°C)*

Despite this very low probability of peak separation, the 19 compounds were sufficiently separated to allow their identification from an injected solution containing 15 ARV and the 4 studied preservatives (cf. **Fig. 4.b**). As predicted, the compounds with similar chromatographic behavior, 3TC, FTC and d4T presented a co-elution at around 3 minutes whereas NFV and SQV had an inversion of peaks order while slightly co-elution at around 18 minutes which correspond to the aforementioned gray zone of uncertainty of prediction.

From the observed results, it can be noticed that the predicted and experimental chromatograms are quite comparable in terms of peaks elution order and retention times (beginning, apex and end) supported by the calculated coefficient of determination (R^2 = 0.9946) between the predicted t_R and observed t_R that was very good demonstrating the accuracy of the prediction of the compounds in the screening method.

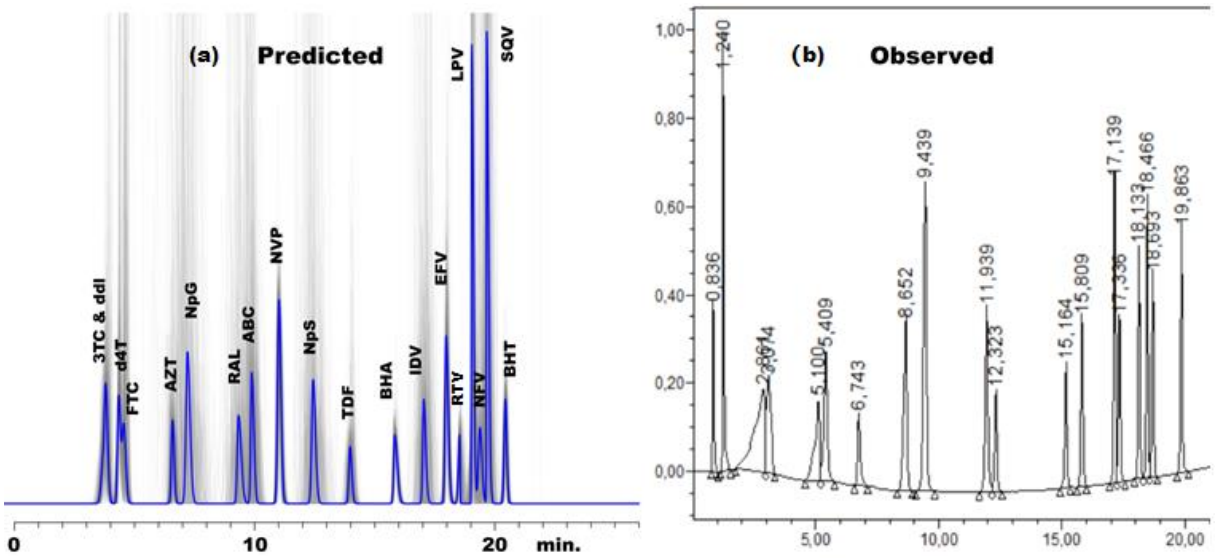

Fig. 4. (a) *Predicted chromatogram for the screening method.* **(b)** *Experimental chromatogram for the screening method on the overall solution*

Legend per elution order in Fig. 4 (b):

(0) Fumaric acid from tenofovir disoproxil fumarate; (1) ddI; (2) 3TC/FTC; (3) d4T; (4) AZT; (5) NpG; (6) RAL; (7) ABC; (8) NVP; (9) NpS; (10) TDF; (11) BHA; (12) IDV; (13) EFV; (14) RTV; (15) LPV; (16) SQV; (17) NFV; (18) BHT

Experimental conditions: Column: XBridge Shield (C18), 100 x 4.6 mm, 3.5 µm; Flow: 1 mL min^{-1}, T°: 35°C, λ = 210 nm, Gradient elution:

For 18 compounds	Methanol (%)	Buffer (%)	Elution phase type
0.00	5	95	Gradient
21.25	95	5	
25.25	95	5	Isocratic plate
25.65	5	95	Rebalancing initial mobile phase
37.25	5	95	

The small changes observed may be explained by the uncertainty of prediction due to the variation of the chromatographic conditions especially on pH factor that was found to have a significant and different influence on the chromatographic behavior of some compounds by relatively changing their t_R. Some relationship observed between pH and t_R were increasing quadratic regression and sigmoid, whereas others were decreasing regression or even no regression at all. The compounds concerned were ddI, 3TC, d4T, AZT, NpG, RAL, ABC, NpS, IDV and NFV at pH > 9 that is the optimal level of pH for the screening method. As a warning to have reproducible results, the analysts should be careful while adjusting the pH of the buffer solution in order to minimize the risk of variation in retention times (beginning,

apex and end) and/or peaks elution order. Other precaution is to have a sufficient re-equilibration time of the column after gradient before a next run as indicated in **Fig. 5**.

We tried also to investigate the probable cause of the fronting peaks observed at 2.86 minutes and at 5.10 minutes. For that, we run separately the solutions of the four sub-groups using the same analytical conditions of the screening method. As can be seen in overlayed chromatograms in **Fig. 6** no peak co-elution was observed inside the sub-group or between sub-groups and no fronting phenomenon occurred. It was noticed that d4T co-elutes with both 3TC and FTC when the three compounds are mixed, but 3TC and FTC are well separated when they are mixed in one solution. On the other hand, the peak of AZT in the chromatogram of sub-group solution 2 was found very good without any fronting peak. We understood that the fronting phenomenon on this analyte in the overall mixture solution is caused by the presence of NpG that co-elutes with AZT in this analytical method. Note that the chromatograms of **Figs. 4.b** and **6** have similar peaks elution order and very closer retention times (at apex).

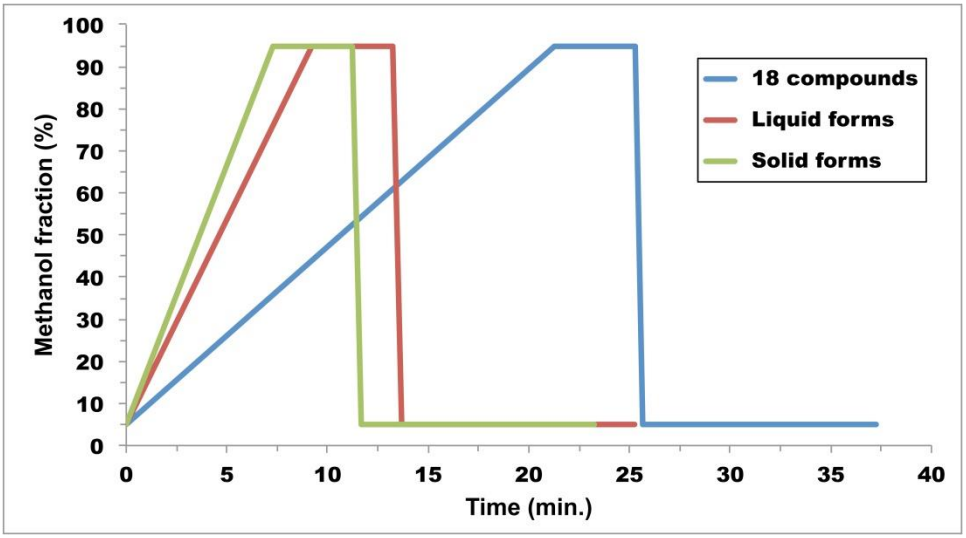

Fig. 5. *Gradient profiles for the screening method of 18 compounds, liquid dosage forms, and solid dosage forms methods*

For 18 compounds	Methanol (%)	Buffer (%)	Elution phase type
0.00	5	95	Gradient
21.25	95	5	
25.25	95	5	Isocratic plate
25.65	5	95	Rebalancing initial mobile phase
37.25	5	95	
For liquid dosage forms	Methanol (%)	Buffer (%)	Elution phase type
0.00	5	95	Gradient
9.25	95	5	
13.25	95	5	Isocratic plate
13.65	5	95	Rebalancing initial mobile phase
25.25	5	95	
For solid dosage forms	Methanol (%)	Buffer (%)	Elution phase type
0.00	5	95	Gradient
7.25	95	5	
11.25	95	5	Isocratic plate
11.65	5	95	Rebalancing initial mobile phase
23.25	5	95	

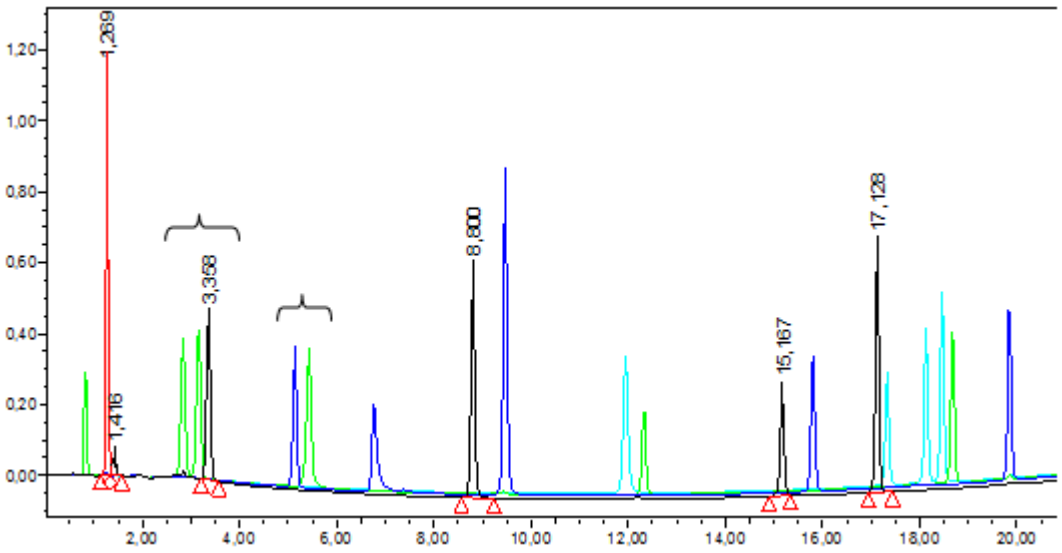

Fig. 6. *Overlaying chromatograms of the 4 sub-groups containing the studied 15 ARV agents and 4 excipients, analysed with the screening method*

Legend per elution order:
Blue peaks belong to the ARV sub-group 2 solution (AZT, RAL, NVP, IDV, BHT);
Green peaks to sub-group 3 (fumaric acid, 3TC, FTC, NpG, TDF, NFV);
Cyan peaks to sub-group 4 (NpS, RTV, LPV, SQV),
Red and Black peaks to sub-group 1 (ddI, d4T, ABC, BHA, EFV)

Experimental conditions: the same as for **Fig. 4**

3.3.2 Second optimization: Generic method dedicated to liquid dosage forms

In a following step, we were interested to the development of methods which are applicable to specific dosage forms containing the studied ARVs. A generic method for the analysis of ARV medicines in liquid dosage forms was optimized at the predicted optimal conditions given in **Table 4**. It was developed on the basis of earlier DoE/DS database on the 3 ARV (3TC, FTC and NFV) and the 4 studied preservatives (BHA, BHT, NpG, NpS) generally used in liquid dosage formulations. These ARV were selected on the basis of their medical prescription in pediatric formulations that are susceptible of containing NpG and NpS as antimicrobial preservatives and/or BHA, BHT as antioxidants, the latter being generally used in the manufacture of medicines that contain fatty compounds or oils to prevent their oxidation.

For this method we focused on the analysis of different liquid dosage forms either in single or combined active ingredients, and on monitoring the content of the four studied excipients in

pediatric formulations such as EPIVIR® (3TC), ZERIT® (d4T), Mezivir® (AZT), VIRAMUNE® (NVP), Ziagen® (ABC), Emtriva® (FTC), and various other ARV medicines containing among others NpG and NpS [21-26]. On the other hand, a recent reflection paper by the European Medicines Agency (EMA) on the use of NpG and NpS as excipients in human medicinal products for oral use stipulated that in oral pharmaceutical formulations, association of those excipients are used with concentrations generally ranging from the authorized content from 0.015 % to 0.2% for NpG and from 0.02% to 0.06% for NpS. Based on the current posology of medicines containing these conservatives, concentrations of 0.2% and 0.06% would correspond to maximal intakes of approximately 140 mg/day and 50 mg/day, respectively [27]. These can serve as acceptable limits of the two antimicrobial preservatives in various formulations. Thus far, to allow monitoring the BHA and BHT excipients in medicines, we considered as specification the acceptable daily intake (ADI) limits fixed by the Joint FAO/WHO Expert Committee on Food Additives. The ADI for BHA was: 0 – 0.5 mg/kg body weight/day, and for BHT: 0 – 0.3 mg/kg body weight/day [28-30] which would be compared to the posology of medicines and the medical prescriptions in order to avoid consuming more than the recommended ADIs. In this way our interest was also to fulfill the above requirements at least by detecting these conservatives. As can be seen in **Table 5** and in **Fig. 7**, the peaks of the ARV are resolved as well of the 4 preservatives in 12 min. The predicted and experimental results were found comparable in terms of peaks elution order except BHA and NFV for which an inversion of peak elution was observed. We noticed a change of more than 1 minute in retention time (at apex) of NpG, NpS and BHA mainly due to the prediction uncertainty and sensitivity of the compounds on slight changes of pH of the aqueous mobile phase.

All ARV components among the most found in liquid dosage forms can be quantified without any interference with the 4 studied excipients. The method for liquid dosage forms is able to detect also the presence of the latter.

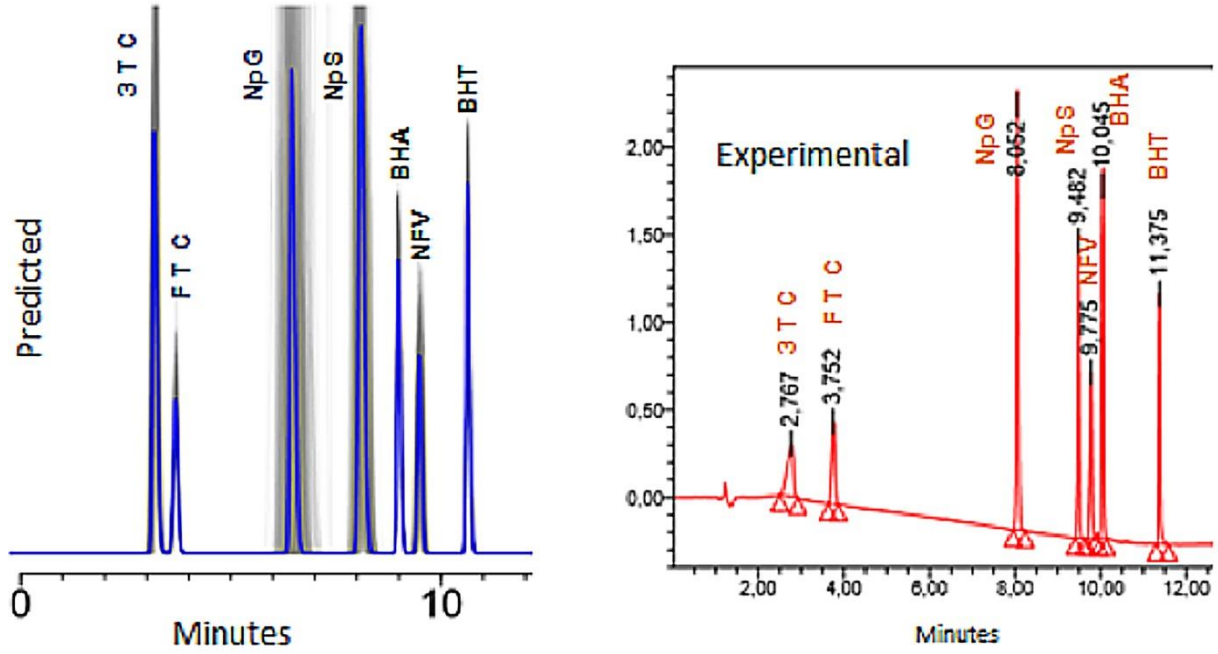

Fig. 7. *Predicted and experimental chromatograms of the method for the analysis of liquid dosage forms*

Experimental conditions: Column: XBridge Shield (C18), 100 x 4.6 mm, 3.5 µm; Flow: 1 mL min^{-1}, T°: 25°C, λ = 210 nm, Gradient elution:

For liquid dosage forms	Methanol (%)	Buffer (%)	Elution phase type
0.0	5	95	Gradient
9.25	95	5	
13.25	95	5	Isocratic plate
13.65	5	95	Rebalancing initial mobile phase
25.25	5	95	

Table 5

Predicted and observed retention times (t_R at apex) on the method for the analysis of liquid dosage forms

Compound	Predicted t_R (min.)	Observed t_R (min.)	Difference (min.)
3TC	3.17	2.77	-0.40
FTC	3.68	3.75	0.07
NpG	6.44	8.05	1.61
NpS	8.09	9.48	1.39
BHA	8.97	10.05	1.08
NFV	9.48	9.78	-0.30
BHT	10.62	11.38	0.76

3.3.3 Third optimization: Generic method dedicated to solid dosage forms

Solid dosage forms are very often used in combination of several ARVs at fixed-dose combinations "FDC" among which are listed 3TC/d4T/NVP, 3TC/AZT/NVP, FTC/TDF/EFV and 3TC/TDF/EFV. In this context, we oriented our third optimization for a generic method starting from the same database previously constructed for all the 19 compounds. The predicted optimal conditions (see **Table 4**) allowed to obtaining the predicted chromatograms for the 4 FDCs (see **Fig. 8**). We tested in practice the conditions of one FDC (i.e. FTC/TDF/EFV) and we obtained the experimental results quite similar in terms of peaks elution order and retention times (at apex) as illustrated in **Fig. 9**.

We noticed that the variation in t_R was less than 0.6 minute, and all peaks were separated, very sharp and symmetric allowing to identify even the fumaric acid at 2.74 minutes. This analyte is released from tenofovir disoproxil fumarate (TDF).

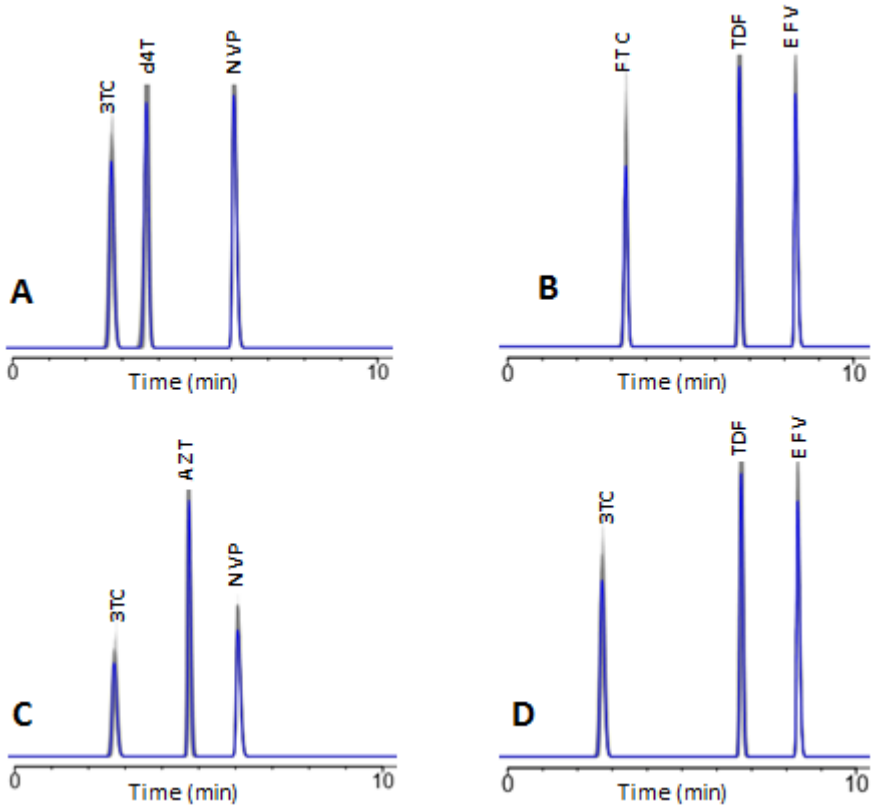

Fig. 8. *Predicted chromatograms of the 4 fixed-dose combinations of 3TC, AZT, d4T, EFV, FTC, NVP, and TDF in solid dosage forms*

Experimental conditions: Column: XBridge Shield (C18), 100 x 4.6 mm, 3.5 µm; Flow: 1 mL min^{-1}, T°: 25°C, λ = 210 nm, Gradient elution:

For solid dosage forms	Methanol (%)	Buffer (%)	Elution phase type
0.0	5	95	Gradient
7.25	95	5	
11.25	95	5	Isocratic plate
11.65	5	95	Rebalancing initial mobile phase
23.25	5	95	

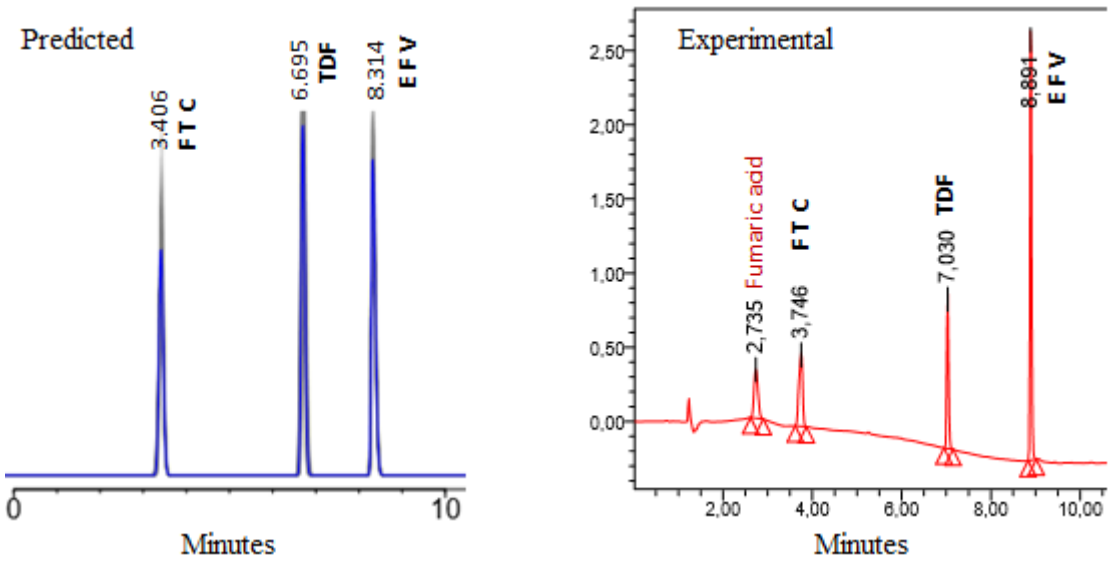

Fig. 9. *Predicted and experimental chromatograms of FTC/TDF/EFV combination. Resolutions are 6 (between fumarate and FTC), 27 (between FTC and TDF), and 24 (between TDF and EFV)*

Experimental conditions: the same as for **Fig. 8**

3.4. Method validation

After the optimization process, it was necessary to demonstrate that the developed analytical methods provide accurate assay results. In this study, we choose the method dedicated to the assay of one FDC composed by Emtricitabine (FTC), Tenofovir (TDF) and Efavirenz (EFV) in tablets formulations. These three molecules were selected for validation since they are used alone or in combination to avoid HIV resistance due to different action mechanisms. In addition, their chemical structures are different and representative of the most popular pharmaco-chemical groups available among the ARV. Emtricitabine belongs to analog of cytidine, tenofovir to analog of nucleotide of adenine and efavirenz is a non-nucleoside of 4-benzyl and 4-benzoyl-3 dimethylamine pyridinones. Indeed, the action of these three compounds is strongly related to their specific structures. Thus it was very interesting to evaluate the ability of method to quantify these compounds through validation. The validation criteria commonly found in the document Q2(R1) of the International Conference on Harmonization (ICH) [15] were considered namely: selectivity, trueness, precision (repeatability and intermediate precision), accuracy, linearity, limit of detection (LOD) / limit of quantitation (LOQ), and dosing range.

At first, we ensured that the selectivity of the method was suitable. Since no interference was observed at the t_R of the peaks of interest here FTC, TDF and EFV, and the resolution between peaks was largely well above 1.5, one can conclude that this validation criteria was fulfilled.

At second, we applied the concept of total error strategy represented by accuracy profiles as decision tool on the fit-for-purpose of the method for its intended use [11-14].

By using the data of CS, the linear regression model was constructed and allowed obtaining the calculated result from VS. The residual sum of square (RSS) values were 4844, 408.5 and 1288 for EFV, FTC and TDF, respectively. Then, accuracy profiles for the three ARV compounds were drawn as can be seen in **Fig. 10** with the results of validation criteria summarized in **Table 6**. The acceptance limits were set at ±10% according to the International Pharmacopoeia which is among the most used pharmacopoeias in Rwanda in the analysis of medicines [31].

From the back-calculated results of VS that are the experimental ones, trueness of the method was assessed as it is the closeness of agreement between a conventionally accepted value or reference value that correspond to the introduced concentrations of the 3 ARV and a mean experimental one. We were able to get information on systematic error that was found acceptable since the relative biases were between - 1.64% and 7.09% for EFV; - 1.38% and 4.02% for FTC; - 4.21% and 4.53% for TDF, all in respect with the acceptable limit of ± 10%.

Method precision was also found acceptable since there was a closeness of agreement among measurements here the back-calculated results of VS obtained from multiple sampling of homogeneous samples of the three ARV. The relative standard deviation values for repeatability and for intermediate precision at the target 100% concentration level were acceptable with a maximum of 1.72 % for EFV, of 2.28% for FTC and of 1.74% for TDF.

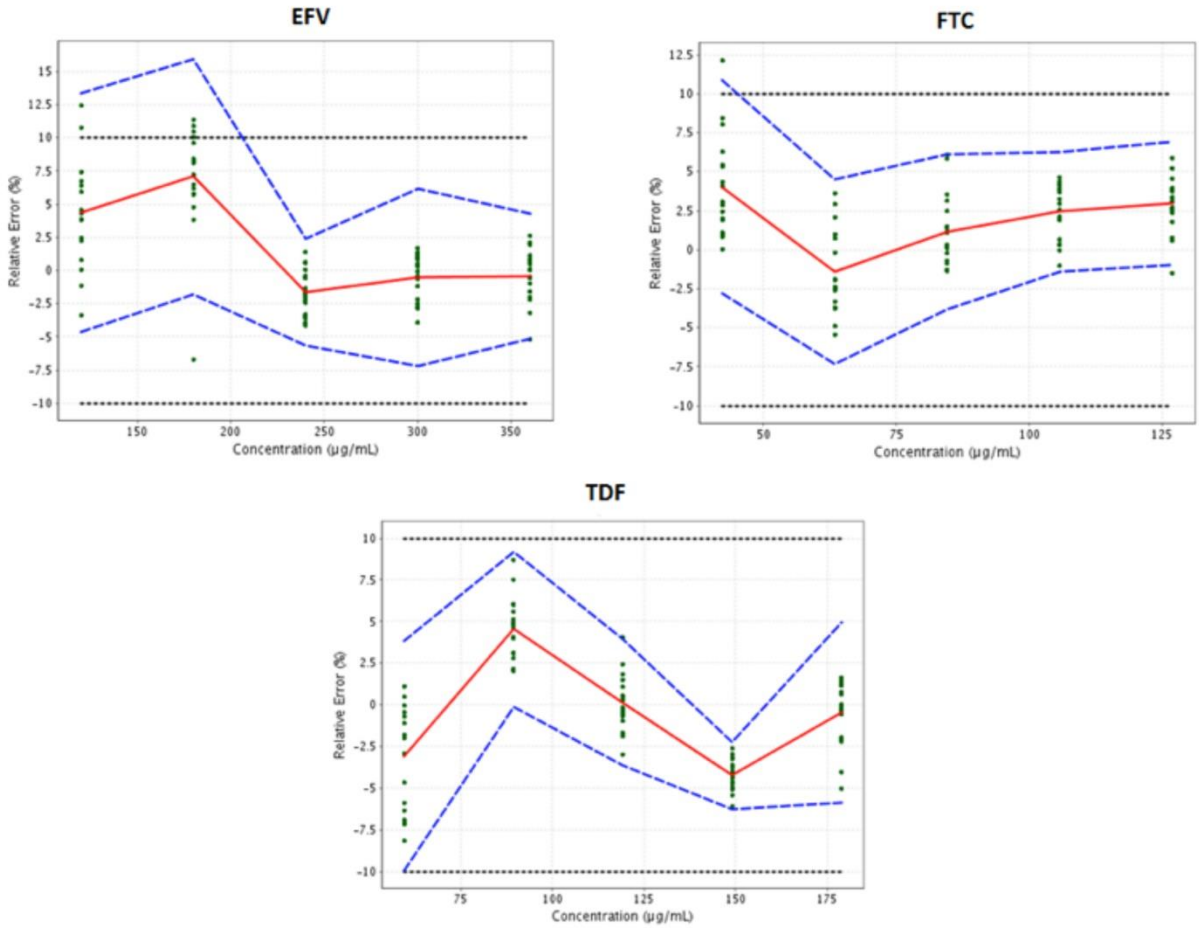

Fig. 10. *Accuracy profiles for quantitative methods validation of EFV, FTC and TDF in tablet formulations. The plain red line represents the relative bias, the dashed lines the 95% β-expectation tolerance limits and the dotted lines the 10% acceptance limits. The dots express the relative error of the back-calculated concentrations plotted with respect to their targeted concentration.*

Table 6

Summary of the validation results

Validation criteria	Conc. (µg mL^{-1})	EFV	Conc. (µg mL^{-1})	FTC	Conc. (µg mL^{-1})	TDF
Trueness: Absolute bias (µg mL^{-1}) & (Relative bias) (%)	*120*	5.23 (4.36)	*42*	1.70 (4.02)	*60*	-1.83 (-3.07)
	180	12.77 (7.09)	*63*	-0.88 (-1.38)	*89*	4.06 (4.53)
	240	-3.95 (-1.64)	*85*	0.99 (1.17)	*119*	0.19 (0.16)
	300	-1.51 (-0.50)	*106*	2.57 (2.43)	*149*	-6.31 (-4.21)
	360	-1.58 (-0.44)	*127*	3.79 (2.99)	*179*	-0.84 (0.47)
Precision: Repeatability (RSD %) / Intermediate precision (RSD %)	*120*	3.82/4.03	*42*	3.15 / 3.15	*60*	3.17 / 3.17
	180	4.08/4.08	*63*	2.72 / 2.72	*89*	1.63 / 1.94
	240	1.52/1.72	*85*	2.28 / 2.28	*119*	1.74 / 1.74
	300	0.97/1.93	*106*	1.75 / 1.75	*149*	0.92 / 0.92
	360	1.82/2.04	*127*	1.82 / 1.82	*179*	1.50 / 2.04
Accuracy: 95% βE (µg mL^{-1}) & (Relative 95%βE) (%)	*120*	114.5−136.1 (-4.62−13.33)	*42*	41.1−46.9 (-2.82−10.85)	*60*	53.7−61.9 (-9.95−3.81)
	180	176.9−208.8 (-1.77−15.95)	*63*	58.8−66.3 (-7.29−4.53)	*89*	89.4−97.7 (-0.12−9.18)
	240	226.6−245.8 (-5.64−2.36)	*85*	81.4−89.8 (-3.78−6.13)	*119*	115.0−124.0 (-3.62−3.94)
	300	278.6−318.7 (-7.19−6.18)	*106*	104.3−112.3 (-1.38−6.24)	*149*	139.8−145.8 (-6.23−2.24)
	360	341.6−375.6 (-5.16−4.28)	*127*	125.7−135.7 (-0.96−6.93)	*179*	168.4−187.8 (-5.87−4.94)
Linearity :	Slope	0.9535	Slope	1.036	Slope	0.972
	Intercept	13.35	Intercept	1.42	Intercept	2.41
	R^2	0.992	R^2	0.995	R^2	0.992
Dosing range :						
Lower LOQ (µg mL^{-1})		206.4	LLOQ	45.14	LLOQ	59.64
Upper LOQ (µg mL^{-1})		360.2	ULOQ	126.9	ULOQ	178.9

βE= β-Expectation Tolerance Interval

In fact, between-run variance equaling or almost equaling to zero means that the intermediate precision equals to repeatability.

Then, to demonstrate the method linearity, we assessed the relationship between the back-calculated results of VS (experimental ones) against the introduced concentrations of the 3 ARV in the samples. The linear regression model was fitted on the two types of concentrations, with a good linearity of the results illustrated (cf. **Table 6**) by the slopes close to 1.

Method accuracy taking into account the total error, *i.e.* systematic and random errors, was assessed from the accuracy profile shown in **Fig. 10**. In addition, as shown in **Table 6**, the relative β-expectation tolerance intervals are in general within a range of [−9.95, 9.18%] except level 1 of FTC, and level 1 and 2 of EFV. As the lower and upper tolerance bounds are included within the acceptance limits for all the targeted concentration levels (except the one mentioned i.e. Level 1 for FTC; level 1 and 2 for EFV), one can ensure that each future result will fall within the acceptable limits with a probability of at least 95% [32].

We estimated also the limit of detection (LOD) that is the smallest quantity of the targeted substance that can be detected, but not accurately quantified in the sample. The reported values were: 6.30; 10.63 and 19.74 µg mL^{-1} for FTC, TDF, and EFV respectively.

The lower limit of quantification (LLOQ) is the smallest quantity of the targeted substance in the sample that can be assayed under experimental conditions with well-defined accuracy meaning in the range of the specified acceptable limits of ±10%. The definition can also be applicable to the upper limit of quantitation (ULOQ) which is the highest quantity of the targeted substance in the sample that can be assayed under experimental conditions with well-defined accuracy in the range of the considered acceptable limits. The limits of quantitation were obtained by calculating the smallest and highest concentrations beyond which the accuracy limits or β-expectation limits go outside the acceptance limits. The intervals between the lower and the upper limits where the procedure achieves adequate accuracy allowed us to set the dosing ranges that were 45.14 - 126.9 µg mL^{-1} for FTC; 59.64 – 178.9 µg mL^{-1} for TDF and 206.4 - 360.2 µg mL^{-1} for EFV.

The uncertainty is a parameter associated with the result of a measurement that characterizes the dispersion of the values that could reasonably be attributed to the measurand. In this study the relative expanded uncertainly (%) have been found less than 8%.

3.5. Application of the method

As the last step of the method lifecycle, the developed and validated method dedicated to solid dosage form was applied in routine for quality control of tablets samples. Two different brand pharmaceutical drugs were tested. They were sampled in Benin and in Rwanda, and claimed to contain FTC, TDF and EFV (sample A) and only FTC and TDF (sample B). The analytical results presented in **Table 7** consisted in the mean percentages of the claimed nominal contents in each medicine and the relative standard deviations calculated on 3 independent samples preparations. Only Brand B satisfied to the acceptable content limits 90.0% - 110.0%. For Brand A, only EFV content was found within these limits whereas the other active ingredients were present but out of the specifications. We can also notice the good precision of the method since the RSD are below 1.74%.

Table 7

Assay results of two ARV medicines coded A and B from Benin and Rwanda. Results consist in the mean percentage of claimed nominal content and the relative standard deviation calculated on 3 independent samples. Specifications are set to 90.0% - 110.0% of the claimed nominal content (mg)

Drug		EFV	FTC	TDF
A	Claimed	600 mg	200 mg	300 mg
	Assay in %	100.3 ± 1.04 %	80.2 ± 0.78 %	84.8 ± 1.17 %
B	Claimed	0 mg	200 mg	300 mg
	Assay in %	-	93.1 ± 1.74 %	91.5 ± 1.40 %

4. CONCLUSION

The main objective of the present study was to develop generic LC methods for qualitative and/or quantitative analysis of 18 ARV namely ABC, ddI, EFV, FTC, IDV, 3TC, LPV, NFV, NVP, RTV, SQV, d4T, TDF, AZT, RAL, ATZ, DRV, ETV and 4 major excipients (NpG, NpS, BHA and BHT) by applying DoE/DS methodology.

Firstly, 15 ARV and the excipients were selected for optimization using D-optimal design based on Gradient time, column temperature and pH of buffer solution selected as analytical factors, the experiments showed that the Gradient time and pH had significant effects on peak separations within the explored experimental domain.

By exploiting the generated DoE database, a screening method was predicted thanks to Monte Carlo simulations for simultaneous analysis of 15+4 compounds using low cost mobile phase composed by methanol and 10 mM ammonium hydrogen carbonate buffer. This method was found useful in the detection of suspected counterfeit/falsified or a mix up of the ARV studied.

Secondly, other methods for the analysis of solid and liquid dosage forms were optimized. For oral solutions or suspensions, a generic method using methanol and 10 mM ammonium formate buffer pH 3.61 was optimized for the analysis of 3DT, AZT, ABC, EFV, FTC, LPV/RTV, etc. together with at least one of the four studied excipients.

Thirdly, for the analysis of solid dosage forms another method was optimized to analyze four tablet fixed-dose combinations. The one for FTC/TDF/EFV was successfully applied in 2 cases: validation and application on real samples. The build DoE/DS database can serve in the prediction of other optimal analytical conditions for the analysis of any other fixed-dose combination or mono-component active ingredient with one or more of the four studied major excipients.

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IV.2 SECTION 2 - Optimization of LC Methods using DoE-DS Database and Geometric Transfer of the methods for Rapid Screening of poor quality antimalarial medicines in Rwanda

(Under submission for publication)

IV.2.1 Preamble to Article 2

Malaria is a major public health concern and one of infant mortality causes in most of developing countries. It is among the top ten causes of death and disability combined in Rwanda. According to the U.S. President's Malaria Initiative (PMI) Malaria Operational Plan for Rwanda, the entire population is at risk of malaria; including an estimated 1.8 million children under five and 443,000 pregnant women (data of 2017). According to the Rwanda Biomedical Center - Malaria and Other Parasitic Diseases Division (RBC/MOPDD data from June 2015), 19 districts out of 30 were classified as high-burden of malaria disease in the country. In 2015, 2,662,706 cases of malaria were reported, and 11 endemic districts accounted for 76% of the cases. On the other hand, the number of related reported annual case fatalities continued to remain relatively unchanged (1.8% - 3.5%, deaths / hospitalized patients) between 2009 and 2015 corresponding to 809, 670, 380, 459, 419, 499, and 489 annual reported deaths respectively [1].

Face to this unhappy situation, the Government of Rwanda has set the management of malaria as key priority in protecting its population. This encouraging initiative is unfortunately confronted to the dramatic battle of medicines counterfeiting for which malaria medications are highly targeted by counterfeiters. Hence, there is need to have rapid and efficient analytical methods that can be used in detecting counterfeit formulations as well as in routine analysis to check the conformity with the required quality specifications.

Instead of building a new DoE-DS database as done for ARVs in the previous section, we exploited an earlier DoE-DS database developed in the Laboratory of Pharmaceutical Analytical Chemistry (ULg, Pharmacy Department) for 19 active ingredients and 4 major

[1] Source: USAID - U.S. President's Malaria Initiative (PMI) Malaria Operational Plan FY 2017 Rwanda. Retrieved May 24, 2018 from https://www.pmi.gov/docs/default-source/default-document-library/malaria-operational-plans/fy17/fy-2017-rwanda-malaria-operational-plan.pdf?sfvrsn=6

excipients. The optimal analytical conditions were predicted for the analysis of 8 active ingredients and 4 major excipients used in Rwanda. Then, after methods optimization, we focused on reducing the analysis time by doing geometric transfer of the methods from long analytical columns to short ones that will enable us increasing the number of samples to be analyzed.

IV.2.2 Article 2

ABSTRACT

An earlier developed design of experiments and design space (DoE-DS) database was exploited to predict and optimize rapid screening methods for the analysis of suspected poor quality antimalarial medicines in Rwanda. The developed methods are specific for the analysis of arteether, artemether, artesunate, lumefantrine and quinine in all dosage forms used in curative treatment, and atovaquone, mefloquine, proguanil used in preventive malaria treatment (chemoprophylaxis), plus four major preservative agents (BHA, BHT, nipagine, and nipasol) generally found in liquid dosage forms.

Optimal analytical conditions and related chromatograms were computed thanks to Monte Carlo simulations and experimented in laboratory to check their fitness for their intended use, and eventually perform some little modifications to obtain better chromatographic results when needed. Then, after optimizing the methods, they were geometrically transferred from long to short analytical columns in order to reduce the analysis time, increase the sample analysis throughput, reduce the mobile phase consumption and budget, and decrease the volume of solvents wastes.

Moreover, the overall screening method was applied to other types of molecules that can be used by counterfeiters and the method showed the ability of detecting several other compounds among other types of antimalarial medicines not officially used in Rwanda, non-steroidal anti-inflammatories (NSAIs), and antibiotics. This test was performed as counterfeiters can use among others wrong active ingredients from classical pharmaceutical compounds in the intention of cheating health professionals and patients, and regulatory officials.

1. INTRODUCTION

Malaria is still a major health concern to tropical countries, especially in the sub-Saharan Africa. Then, antimalarial medicines are fundamental to any strategy for effective reduction of morbidity and mortality caused by that disease. According to the latest estimates of the World Health Organization (WHO), released in end of 2016, there were 212 million cases of malaria in 2015, and 429,000 deaths. Moreover, according to other WHO estimates, most of cases in 2015 were in the WHO African Region (90%), followed by the WHO South-East Asia Region (7%) and the WHO Eastern Mediterranean Region (2%) [1-2].

On the other hand, before placing any medicine on the market, National Medicines Regulatory Authorities (NMRA) check its compliance to the compulsory requirements for safety, efficacy and quality. That control normally involves the assessment of related documentation supported by inspections on compliance with Good Manufacturing Practices (GMP). Then, adherence to GMP requirements during product manufacture ensures that each distributed batch will constantly comply with the required specifications.

However, the illicit trade of poor quality medicines constitutes a very serious threat to the public health worldwide, and particularly in developing countries where drug regulatory systems are weak and controls very limited [3-4]. On the other hand, the quality of genuine medicines may be easily deteriorated through improper handling and poor storage conditions; therefore, there is need of having efficient analytical methods that can serve in routine analysis of different pharmaceutical products especially when analyzing suspected poor quality products that may contain no active ingredients, wrong ingredients, sub-degradation products and impurities, etc.

In this context, we have developed a set of simple and rapid HPLC methods for the screening of antimalarial medicines used in Rwanda by exploiting an earlier developed design of experiments and design space (DoE-DS) database created by Debrus et al. (2011) [5], and we performed geometric transfers from long to shorter analytical columns in order to reduce the analysis time, increase the sample analysis throughput, and reduce the mobile phase consumption as well as saving cost of the later. The same methods could be applied by other countries in the analysis of similar antimalarial medicines whenever needed.

Design of experiments (DoE) is a powerful research methodological approach used in various industrial fields including the pharmaceutical sector while conducting different types of experiments to develop new products and processes, and to improve existing products and processes faster. In pharmaceutical analysis, it presents advantages of allowing efficient exploration of different chromatographic factors effects and interactions between them; and when applying it correctly, DoE will allow to reduce the development and production costs, and to improve quality and reliability. DoE is much more rigorous and advantageous than traditional methods of experimentation such as one-factor-at-a-time and expert trial-and-error. That proficient approach allows practitioners to explicitly model the relationship among the studied variables in any system, and to ultimately arrive at better solutions in short time [6 - 9].

On the other hand, design space (DS) defined as a "multidimensional combination and interaction of input variables that have been demonstrated to provide assurance of quality" [9], is suitable for the prediction of optimal analytical conditions within the experimental domain. Therefore, in the framework of separation methods development, DS can be clearly considered as a zone of theoretical robustness since modifications of the method parameters will not significantly affect the separation quality. Consequently, the combination of design of experiments and design space methodologies (DoE-DS) is highly important while optimizing the operating conditions governing a separation process [10].

The liquid chromatography (LC) has been privileged as a conventional analytical technique widely used in pharmaceutical analysis and pharmacopoeias, but also by its capacity to analyze several components simultaneously. Moreover, the number of HPLC equipment is increasing in Rwanda, especially in university laboratories and Government institutions dealing with medicines.

Hence, the targeted medicines are those manufactured in one or combined active ingredients among those illustrated in **Fig. 1**: arteether (AE), artemether (AM), artesunate (AS), atovaquone (AT), lumefantrine (LF), mefloquine (MF), proguanil (PG), and quinine (QN), plus four commonly used excipients as antioxidants or antimicrobial preservatives : butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), nipagine (NpG), and nipasol (NpS) mostly found in liquid dosage formulations.

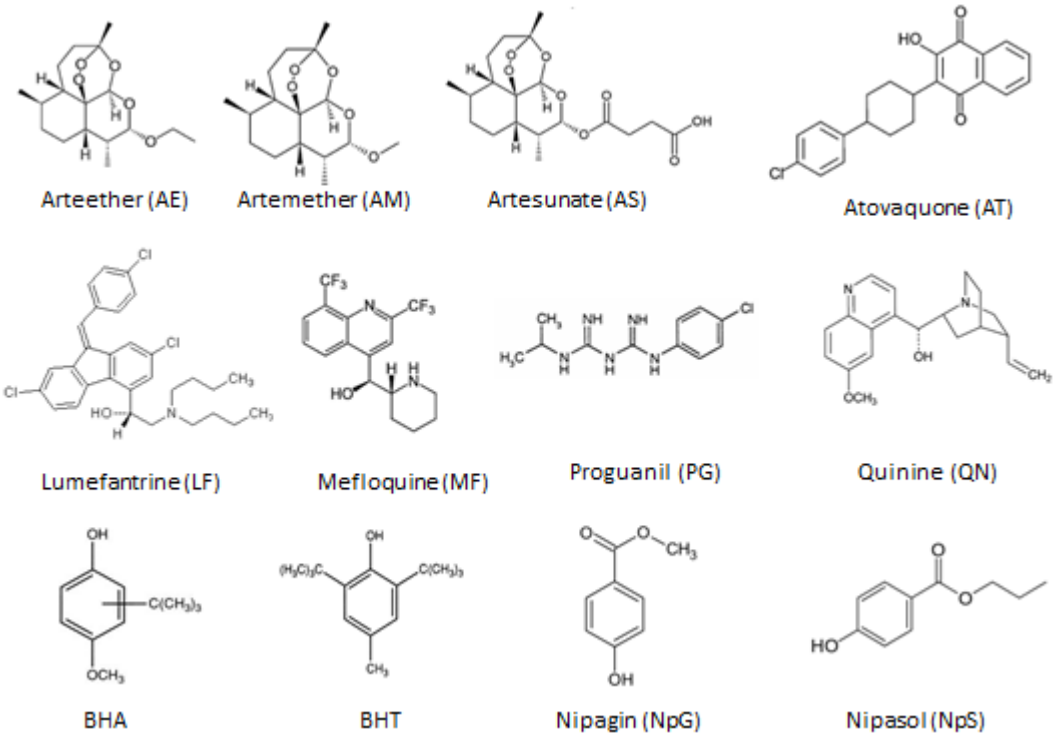

Fig. 1 *Molecular structures of the 8 antimalarial medicines and 4 major excipients*

Furthermore, the developed methods will be tested to other pharmaceutical substances in order to assess their ability in detecting other pharmaceutical compounds than the studied ones, and therefore ensure that the methods can detect undeclared active and/or inactive ingredients in poor quality medicines. Hence, the following substances were tested with the screening method for that purpose: (i) other antimalarial medicines not considered during methods development and not officially used in malaria treatment in Rwanda: amodiaquine, artemisinine, chloroquine, cinchonine, dihydroartemisinine, halofantrine, piperaquine, primaquine, pyrimethamine, sulfadoxine, and sulfalene; (ii) anti-inflammatories and associated drugs: aspirin, atenolol, caffeine, codeine, dexamethazone, diazepam, diclofenac, furosemide, ibuprofen, metamisole (dipyrone), methylpredinizolone, naproxen, paracetamol, and prednizolone; (iii) antibiotics: albendazole, amoxicillin, ampicillin, cefotaxime, chloramphenicol, clindamycin, levofloxacin, penicillin-V, sulbactam, tazobactam, and trimethoprim; and (iv) finally metronidazole, sodium benzoate, and lidocaine.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and drug samples used during methods development and application

Arteether (100%), Artemether (100%), Artesunate (> 97%), and Lumefantrine (100%) were purchased from SensaPharm (Lanchester, UK), Atovaquone (> 99%) and Mefloquine hydrochloride (98.8%) from Alsachim (Illkirch, France), Butylated hydroxyanisole (BHA) (96%) and Nipagine (Methyl paraben) (99%) from Alfa Aesar GmbH & Co KG (Karlsruhe, Germany), Butylated hydroxytoluene (BHT) (≥ 99%) from Merck Schuchardt OHG (Hohenbrunn, Germany), Nipasol (Propyl paraben) (101.9%) and quinine sulfate (99.0%) from Fagron N.V. (Waregem, Belgium), quinine dihydrochloride (100.8%) from Molekula Limited (Dorset, UK), Proguanil hydrochloride (99.8%), Hydrochloric acid (37%) and ammonium formate (98.1%) from VWR International BVBA (Leuven, Belgium), Methanol LC grade from Avantor Performance Materials B.V. (Deventer, The Netherlands), the Ultrapure Water was produced with a Milli-Q Plus 185 water purification system (Millipore, Billerica, MA, USA), and various samples of quinine tablets 300 mg, quinine for injection 300 mg/mL and 600 mg/mL as labelled on the marketed products, artemether-lumefantrine tablets 20 mg / 120 mg, artesunate powder for injection 60 mg, mefloquine 250 mg tablets, and atovaquone-proguanil 62.5 mg / 25 mg were purchased from different pharmacies in Rwanda.

2.1.2 Chemicals used in the assessment of the capacity of the screening method in detecting other components

On the other hand, other commonly used pharmaceutical ingredients were selected and involved in the study to check the ability of the new LC methods in detecting other medicines rather than the principal 12 analytes considered during methods development, and therefore evaluate the capacity of the methods to screen other substances in case of counterfeiting. Hence, eleven (11) analytes among the category of antimalarial medicines not used in Rwanda and related substances were checked with the methods. Those are amodiaquine hydrochloride (99%), chloroquine diphosphate (98%), cinchonine (99.9%), pyrimethamine

(99.0%), and sulfadoxine (99.9%) purchased from Sigma Aldrich (St. Louis, MO, USA), artemisinine (99.6%) and dihydroartemisinine (100.0%) from Apoteket AB (Stockholm, Sweden), halofantrine was gratefully provided by GlaxoSmithKline (Genval, Belgium), piperaquine was extracted from the P-Gvaxin formulation from Bliss GVS Pharma (Mumbai, India), primaquine diphosphate (98%) and sulfalene "Ph.Eur" from Fagron N.V. (Waregem, Belgium); then, were also checked fifteen (15) anti-inflammatories and associated molecules which are acetylsalicylic acid (99.9%), caffeine (100.1%), ibuprofen (99.6%), furosemide (100.5%), metamizole sodium i.e. dipyrone "Ph.Eur", methylprednisolone "Ph.Eur", prednisolone (100.7%), and paracetamol (99.5%) purchased from Fagron N.V. (Waregem, Belgium), atenolol "Ph.Eur", codeine phosphate "Ph.Eur", dexamethasone "Ph.Eur", diazepam "Ph.Eur", diclofenac (99.7%), naproxen (> 98%), and sodium benzoate (99.9%) from Sigma Aldrich (Bornem, Belgium); thirteen (13) antibiotics and antiparasitics: amoxicillin (99.1%), clindamycin (95.8%), chloramphenicol "Ph.Eur", ciprofloxacin (99.6%), metronidazole (99.9%), phenoxymethylpenicillin (or Penicillin V) (100.2%), sulbactam (91.5%), tazobactam (99.2%), tetracycline (96.6%), and trimethoprim (99.2%) purchased from Fagron N.V. (Waregem, Belgium), ampicillin (98.0%) from AppliChemBiomedica (Darmstadt, Germany), cefotaxime (94.3%), Ceftriaxone (93.9%), and levofloxacin (99.0%) from Molekula Limited (Dorset, UK).

2.2 Methods development strategy and validation

The screening and sub-screening methods in gradient mode were developed thanks to an existing DoE/DS database created by Debrus et al. (2011). These methods were developed through different steps illustrated in **Fig. 2**, and to each method were computed the optimum analytical conditions and related chromatograms thanks to Monte Carlo simulations.

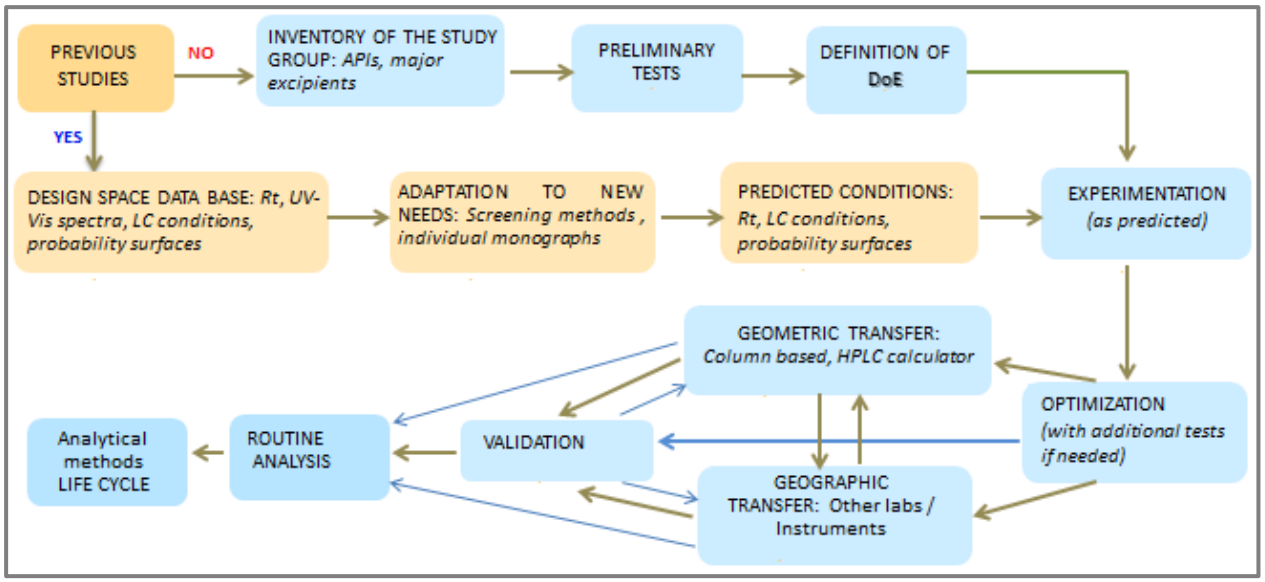

Fig. 2 *Key steps applied in developing new methods*

Indeed, the 12 analytes were split into four subgroup categories of compounds that can be analyzed together as illustrated in **Fig. 3**. Then, to each subgroup was computed a generic method for the analysis of all its compounds when detecting suspected counterfeit drugs.

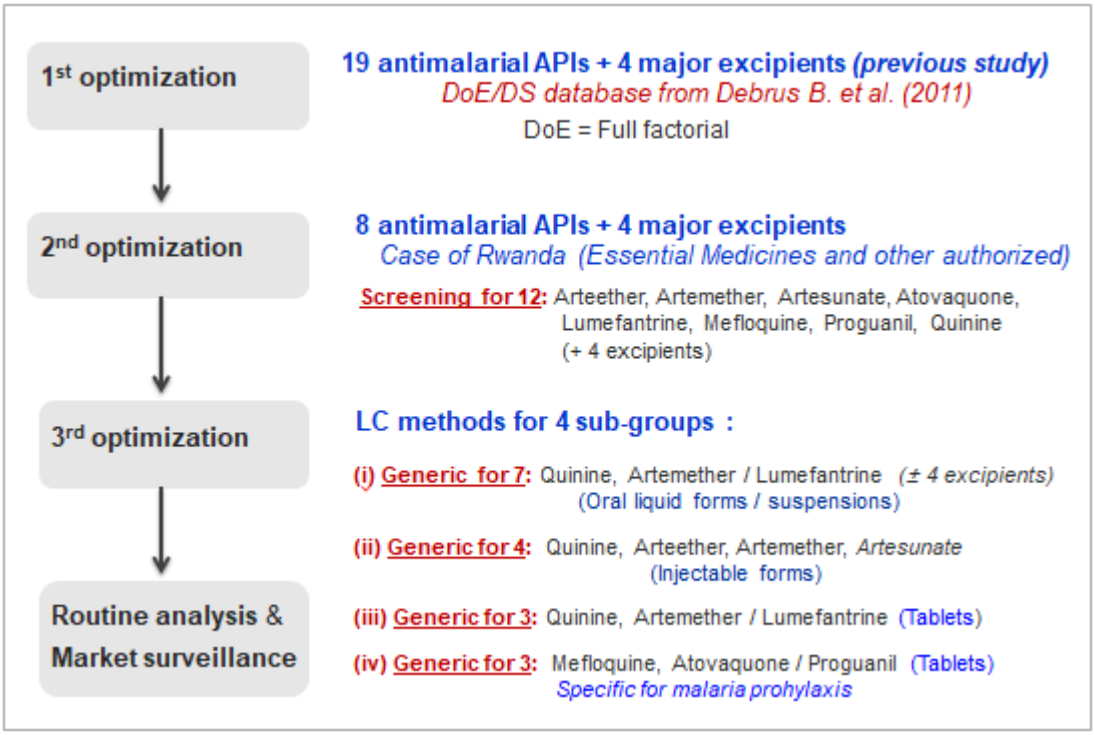

Fig. 3 *Chronological optimization process*

Moreover, to check the ability of the overall screening method in detecting other compounds that can be used by counterfeiters instead of genuine medicines we analyzed other compounds that are widely available on the market.

2.3 Instrumental and software

2.3.1 HPLC equipment

The methods were developed in Belgium on a Waters 2695 Alliance HPLC Separation Module coupled to Waters 2996 photodiode array (PDA) detector from Waters Corporation (Milford, MA, USA) piloted with Empower 2.0 software (Waters Corporation), and transferred to Rwanda on three different LC systems namely Prominence UFLC equipped with a UV detector from Shimadzu South Africa (Pty) Ltd (Johannesburg, SA), Agilent 1200 series equipped with a diode array detector (DAD) from Agilent Technologies (Böblingen, Germany), and Cecil HPLC Adept Series equipped with a UV detector from CECIL Instruments Limited (Cambridge, UK), but in this paper we are going to limit on reporting the different strategies and results obtained in the originator laboratory.

Then, different chromatographic columns packed with C18 stationary phase, in different dimensions (L: 250 mm to 50 mm, ID: 4.6 mm; dp: 5 µm and 3.5 µm) were used during the methods development and geometric transfer as described in the experimental section.

2.3.2 Softwares

Empower 2.0 software (Waters Corporation, MA, USA) for Windows was used to control the Waters Alliance HPLC system, to record the signals from the detector and interpret the generated chromatograms. Different gradients for the geometric transfer from columns to others were calculated using HPLC Calculator v3.0 developed by Guillarme et al. [11].

Then, JMP 12.2.0 (SAS Institute Inc., NC, USA) and R 3.2.2 (GNU project / Free Software Foundation, MA, USA) softwares were used for generating the DoE trials and experimental data treatment.

2.4 Preparation of sample solutions

2.4.1 Sample solutions for the screening and sub-screening methods

A) Solution S12: Sample solution for overall screening method of 12 analytes

A mixture of 12 analytes (8 active ingredients and 4 excipients) was used and prepared as follows: In the first step approximately 10.0 mg of atovaquone, lumefantrine, mefloquine, proguanil, quinine sulfate, BHA, BHT, nipagin, and nipasol were weighed in 100.0 mL volumetric flask and dissolved in methanol under 10 minutes of ultrasonic bath for non-easily soluble, then completed to volume with the same solvent. This solution was annotated *"Solution S9"* containing about 100 µg mL^{-1} of each analyte. In the second step, approximately 10.0 mg of each of the arteether, artemether and artesunate were weighed in 2.0 mL volumetric flask, and dissolved with *Solution S9*. This was the final solution annotated *"Solution S12"* to be injected in the chromatographic system. It contained around 100 µg mL^{-1} of the first nine analytes, and 5,000 µg mL^{-1} of the last three analytes.

B) Solution S7: Sample preparation for the screening method of quinine, and artemether/lumefantrine in oral liquid formulations with BHA, BHT, NpG and NpS

A mixture of 7 analytes (3 active ingredients and 4 excipients) was used and prepared as follows: In the first step approximately 10.0 mg of lumefantrine, quinine sulfate, BHA, BHT, nipagin and nipasol were weighed in 100.0 mL volumetric flask and dissolved in methanol under 10 minutes of ultrasonic bath, and then completed to volume with the same solvent. This solution was annotated *"Solution S6"* containing about 100 µg mL^{-1} of each analyte. Then, in the second step, approximately 10.0 mg of artemether were weighed in a 2.0 mL volumetric flask and dissolved with Solution 6. This was the final solution annotated *"Solution S7"* to be injected in the chromatographic system. It contained around 100 µg mL^{-1} of the first six analytes, and 5,000 µg mL^{-1} of artemether.

C) Solution Q3A: Sample preparation for the screening method of arteether, artemether, artesunate, and quinine injectable formulations

In the first step, 10.0 mg of quinine base in quinine dihydrochloride were dissolved with methanol in 100.0 mL volumetric flask; and in the second step, this solution was used to dissolve and dilute 10.0 mg of each of the arteether, artemether and artesunate weighed in a 2.0 mL volumetric flask. This was the final solution annotated *"Solution Q3A"* to be injected in the chromatographic system. It contained around 100 µg mL^{-1} of quinine, and 5,000 µg mL^{-1} of the three artemisinin compounds.

D) Solution 3/QAL: Sample preparation for the screening method of quinine, and artemether/lumefantrine in tablets formulations

In the first step, a quantity containing 10.0 mg of quinine base in quinine sulfate, and 10.0 mg of lumefantrine were weighed in 100.0 mL volumetric flask and dissolved in methanol under 10 minutes of ultrasonic bath, and then completed to volume with the same solvent. In the second step, this solution was used to dissolve 10.0 mg of artemether weighed in 2.0 mL volumetric flask. This was the final solution annotated *"Solution 3/QAL"* to be injected in the chromatographic system. It contained about 100 µg mL^{-1} of the first two analytes, and 5,000 µg mL^{-1} of artemether.

E) Solution 3/MPA: Sample preparation for the screening method of mefloquine, proguanil/atovaquone in tablets formulations

10.0 mg of mefloquine, proguanil and atovaquone were weighed in 100.0 mL volumetric flask and dissolved in methanol under 10 minutes of ultrasonic bath, then completed to volume with the same solvent. This solution was annotated *"Solution MPA"* to be injected in the chromatographic system. It contained about 100 µg mL^{-1} of each analyte.

2.4.2 Preparation of miscellaneous sample solutions to check the ability of the methods in detecting other compounds

A) Other antimalarial medicines

The following sample solutions of other antimalarial active ingredients not used in Rwanda were prepared to assess the ability of the screening method in detecting them in case they were fraudulently used by counterfeiters or accidently (but rarely) by authentic manufacturers as sub-standards. Methanol was used as unique solvent as done in the previous section.

(i) Solution ART: Composed by artemisinine solution at around 5,000 µg mL^{-1} by dissolving approximately 10.0 mg in 2.0 mL volumetric flask;

(ii) Solution DHA: Composed by dihydroartemisinine solution at around 5,000 µg mL^{-1} prepared in the same conditions as for artemisinine;

(iii) Solution ACP: Composed by amodiaquine, chloroquine, and piperaquine at around 250 µg mL^{-1} by dissolving approximately 5.0 mg of each analyte in 20.0 mL volumetric flask;

(iv) Solution CPS: Composed by cinchonine, pyrimethamine, and sulfadoxine at around 250 µg mL^{-1} and prepared in same conditions as for Solution ACP;

(v) Solution HPS: Composed by halofantrine, primaquine (newly introduced in the list of antimalarial essential medicines in Rwanda [12]), and sulfalene at around 250 µg mL^{-1} in the same conditions as for Solution CPS.

B) Non-steroidal anti-inflammatory (NSAI) and related compounds

NSAI and related compounds were prepared in methanol too by dissolving 5.0 mg of different selected substances in 20.0 mL volumetric flasks as follows:

(i) ***Solution NSAI 1:*** Composed by diclofenac, caffeine, acetylsalicylic acid, and ibuprofen;

(ii) ***Solution NSAI 2:*** Composed by dexamethasone, atenolol, sodium benzoate, and paracetamol;

(iii) ***Solution NSAI 3:*** Composed by codeine, naproxen, prednisolone, and metamisole;

(iv) ***Solution NSAI 4:*** Composed by diazepam, furosemide, and methylprednisolone.

C) Antibiotics sample solutions

Antibiotic compounds generally supplied as generic medicines, were also prepared in methanol by dissolving 5.0 mg of each selected compound in 20.0 mL volumetric flask and diluted with the same solvent as follows:

(i) ***Solution ANTB 1:*** Composed by ceftriaxone, cefotaxime, ampicillin, and amoxicillin;
(ii) ***Solution ANTB 2:*** Composed by chloramphenicol, levofloxacin, penicillin V, and trimethoprim;
(iii) ***Solution ANTB 3:*** Composed by clindamycin, sulbactam, and tazobactam;
(iv) ***Solution ANTB 4:*** Composed by tetracycline, and ciprofloxacin.

3. EXPERIMENTATION

3.1 Overall screening method

An overall screening method for the analysis of 8 active ingredients and 4 excipients was developed by exploiting an earlier created DoE-DS database and computing suitable and optimal analytical methods as described in **Table 1** dedicated to the studied analytes. Hence, this method is applicable to any antimalarial medicine claimed to contain AE, AM, AS, AT, BHA, BHT, LF, MF, NpG, NpS, PG, and QN, and can help in detecting any counterfeit formulation composed by one or more of these analytes.

Table 1

Predicted and geometric analytical conditions for the screening method applicable to the analysis of suspected counterfeit antimalarial medicines claimed to contain one or more of the following 12 analytes: AE, AM, AS, AT, BHA, BHT, LF, MF, NpG, NpS, PG, and QN

<table>
<tr><td>Columns:</td><td>(i) XBridge C18, 250 x 4.6 mm, dp: 5µm with a guard column (XBridge C18, 5 x 4.6 mm, dp: 5 µm);
(ii) SunFire C18, 250 x 4.6 mm, dp: 5µm;
(iii) Zorbax Extend C18, 150 x 4.6 mm, dp: 5 µm</td></tr>
<tr><td>Mobile phase:</td><td>a) Methanol LC grade and 10 mM ammonium acetate buffer pH 5.65 adjusted with 6N HCl or acetic acid.
b) Methanol LC grade and 10 mM ammonium formate buffer pH 2.5 adjusted with 6N HCl or formic acid.</td></tr>
<tr><td>Elution mode:</td><td>Gradient:
<table>
<tr><td rowspan="2">250x4.6 mm
Time (min)</td><td rowspan="2">150x4.6 mm
Time (min)</td><td colspan="2">MeOH
(%, v/v)</td><td colspan="2">Buffer
(%, v/v)</td></tr>
<tr><td>Test 1</td><td>Test 2</td><td>Test 1</td><td>Test 2</td></tr>
<tr><td>00.0</td><td>00.0</td><td>5</td><td>10</td><td>95</td><td>90</td></tr>
<tr><td>60.0</td><td>35.5</td><td>95</td><td>90</td><td>5</td><td>10</td></tr>
<tr><td>70.0</td><td>41.5</td><td>95</td><td>90</td><td>5</td><td>10</td></tr>
<tr><td>71.0</td><td>42.1</td><td>5</td><td>5</td><td>95</td><td>90</td></tr>
<tr><td>100.0</td><td>59.5</td><td>5</td><td>5</td><td>95</td><td>90</td></tr>
</table></td></tr>
<tr><td>Temperature:</td><td>35° C</td></tr>
<tr><td>Flow rate:</td><td>1 mL min^{-1}</td></tr>
<tr><td>Wavelength:</td><td>230 nm</td></tr>
<tr><td>Injected volume:</td><td>10 µL</td></tr>
</table>

3.2 Sub-screening methods

After optimization of the overall screening method for the analysis of 8 active ingredients and 4 excipients, we split the 8 active ingredients into sub-group categories as per the existing main pharmaceutical forms and treatment mode for both curative and prophylaxis medications. Hence, we found four sub-group categories composed by *(i) Quinine, artemether / lumefantrine* in oral liquid formulations that may contain the four preservatives and antioxidants (BHA, BHT, NpG, and NpS), *(ii) Quinine, arteether*, *artemether*, and *artesunate* in injectable forms, *(iii) quinine*, and *artemether / lumefantrine* in tablet forms, and *(iv) mefloquine, atovaquone / proguanil* which are used as prophylaxis medications and generally found in tablet forms. Then, the computed optimal analytical conditions are summarized in **Tables 2 - 3** for the four sub-group categories.

Table 2

Predicted sub-screening method applicable to the analysis of suspected counterfeit antimalarial medicines claimed to contain QN, and AM / LF in oral liquid forms containing one or more of the 4 studied excipients (BHA, BHT, NpG, NpS)

Columns:	*(i) XBridge C18, 250 x 4.6 mm, dp: 5µm with a guard column (XBridge C18, 5 x 4.6 mm, dp: 5 µm);* *(ii) SunFire C18, 250 x 4.6 mm, dp: 5µm;* *(iii) Zorbax Extend C18, 150 x 4.6 mm, dp: 5 µm*
Mobile phase:	Methanol LC grade and 10 mM ammonium formate buffer pH 4.3 adjusted with formic acid or 6N HCl
Elution mode:	Gradient:

250x4.6 mm Time (min)	150x4.6 mm Time (min	MeOH (%, v/v)	Buffer (%, v/v)
00.0	00.0	5	95
20.0	11.5	95	5
30.0	17.5	95	5
31.0	18.1	5	95
60.0	35.5	5	95

Temperature:	25° C
Flow rate:	1 mL min^{-1}
Wavelength:	230 nm
Injected volume:	10 µL

Table 3

Predicted sub-screening method applicable to the analysis of suspected counterfeit antimalarial medicines claimed to contain (a) QN, AE, AM and AS in injectable forms, (b) QN, and AM / LF in tablet forms, and (c) MF, AT / PG used in prophylaxis treatment

Columns:	*(i) XBridge Shield RP18, 100 x 4.6 mm, dp: 3.5µm;* *(ii) XTerra RP18, 50 x 4.6 mm, dp: 3.5µm;* *(iii) Zorbax Extend C18, 50 x 4.6 mm, dp: 5 µm*
Mobile phase:	Methanol LC grade and 10 mM ammonium formate buffer pH 2.5 adjusted with formic acid or 6N HCl
Elution mode:	Gradient:

100x4.6 mm Time (min)	50x4.6 mm Time (min)	MeOH (%, v/v)	Buffer (%, v/v)
00.0	00.0	5	95
23.25	11.0	95	5
27.25	13.0	95	5
27.65	13.2	5	95
39.25	19.0	5	95

Temperature:	25° C
Flow rate:	1 mL min^{-1}
Wavelength:	230 nm
Injected volume:	10 µL

4. RESULTS AND DISCUSSIONS

4.1 Influence of factors on the chromatographic behavior of the analytes

As illustrated in **Fig. 4** (A, B, C); from the modelled retention times vs. the studied experimental factors (TG, pH, and T°), one can notice that the tR is significantly dependent to the variation of the TG and pH of the mobile phase, while the impact of temperature is not significant between 25°C and 35°C. Then, as normally known, the smaller the TG is, the shorter the analysis time is and consequently the t_R of the analytes. Concerning pH variation, it was noticed that some compounds such as quinine (QN), artesunate (AS), mefloquine (MF), atovaquone (AT), and lumefantrine (LF) are susceptible of sensitive changes of t_R at certain pH levels while others are relatively stable. For example it was noticed that the t_R of QN and LF increase with the variation of pH from lower to higher pH levels; and an opposite situation was observed for AS and AT where the t_R decreases from lower to higher pH levels. On the other hand, the t_R of MF is stable at pH < 5 and increases at pH > 5. The other compounds i.e. nipagine (NpG), nipasol (NpS), proguanil (PG), BHA, artemether (AM), arteether (AE) and BHT have a stable t_R in comparison with the variation of pH levels within the studied range of pH 2.5 – 7.0. One should also notice that at certain pH levels some compounds would co-elute such as NpG and QN at around pH 2.8; NpS and PG at around pH 2.5; BHA and LF at around pH 3.2; MF and AS at around pH 5.2; and BHT, AE, AT at around pH 6.4 depending on physicochemical properties of each analyte.

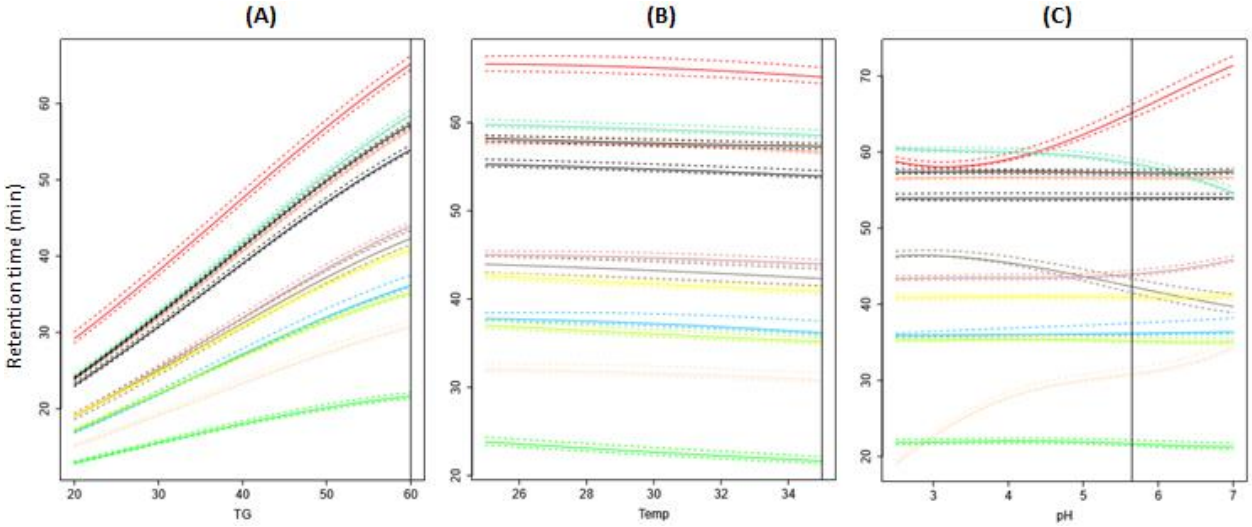

Fig. 4 *Modelled retention times (t_R) of different compounds with respect to factors of experimental design:* ***(A)*** *TG;* ***(B)*** *T° (°C);* ***(C)*** *pH*

4.2 DoE/DS prediction and related experimental results

The strategy of design of experiments (DoE) was previously applied by Debrus B. et al. (2011) [5], and the database created in that study helped to predict the best analytical conditions for (i) *Simultaneous separation of arteether (AE), artemether (AM), artesunate (AS), atovaquone (AT), lumefantrine (LF), mefloquine (MF), proguanil (PG), quinine (QN), BHA, BHT, nipagin (NpG),* and *nipasol (NpS)* for the analysis of these compounds in suspected counterfeit antimalarial medicines, (ii) *Simultaneous separation of AM, LF, QN, BHA, BHT, NpG, and NpS* for the analysis of QN or AM / LF in liquid dosage forms generally containing the four excipients, (iii) *Simultaneous separation of AE, AM, AS, and QN in injectable formulations* such as powders for injection or aqueous and oily forms, and (iv) *Simultaneous separation of AT, MF and PG in tablet forms of the three prophylactic antimalarial medicines.* In fact, in real life, these methods shall be used generally in the analysis of suspected counterfeit medicines, and each method shall be selected according to the type of samples to be analyzed.

Thus far, the predicted optimal analytical conditions are given in **Tables 1-3** for each category of analytes, and the **Fig. 5 - 6** illustrate the probability surfaces showing the zone(s) of robustness (i.e. DS) within which the separation of the studied analytes are optimal for the screening and sub-screening methods of the 12 and 7 analytes respectively. The related predicted and experimental chromatograms are illustrated in **Fig. 7 - 8**.

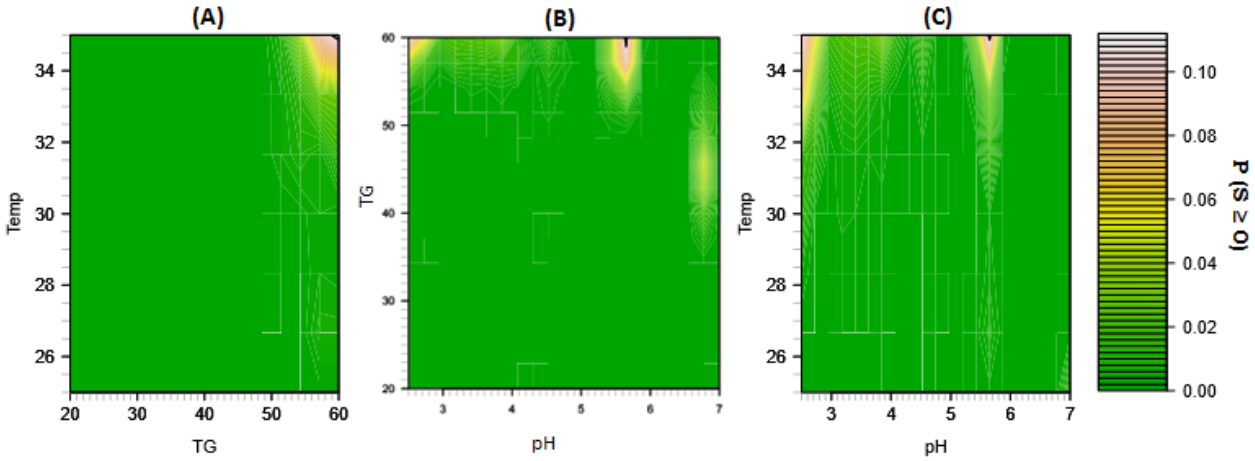

Fig. 5 *Probability surfaces to reach S ≥ 0:* ***(A)*** *TG vs. Temp.;* ***(B)*** *pH vs. TG;* ***(C)*** *pH vs. Temp. for the screening method of 12 analytes*

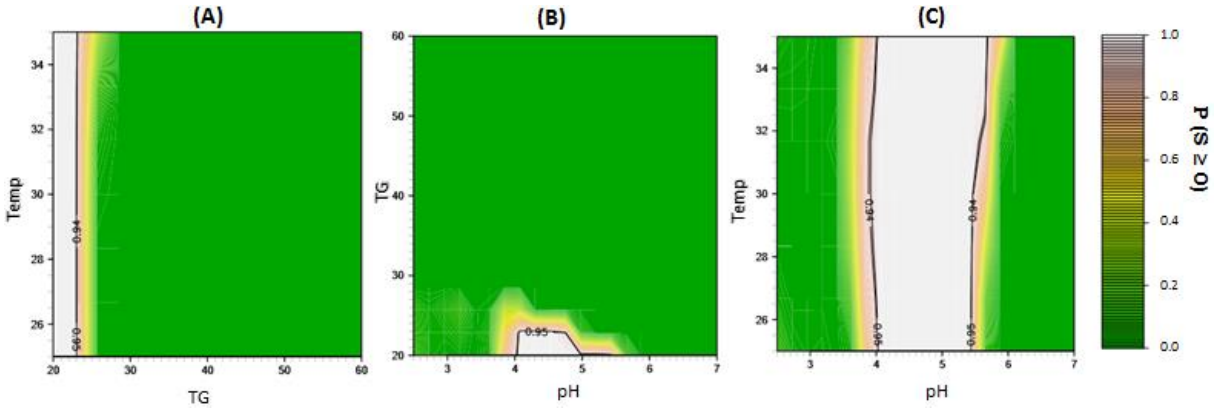

Fig. 6 *Probability surfaces to reach S ≥ 0:* ***(A)*** *TG vs. Temp.;* ***(B)*** *pH vs. TG;* ***(C)*** *pH vs. Temp. for the sub-screening method of 7 analytes*

In fact, when the probability of separation P(S > 0) is high, the risk of peak co-elution is low for the studied analytes. In this study for example, the optimal conditions for simultaneous analysis of QN and AM/LF in liquid dosage forms including the 4 excipients has a very good probability of more than 94% to separate well the seven analytes while the screening method for the 12 analytes has a lower probability of around 10% for separating the 12 analytes due to compounds such as NpS and PG eluting at around t_R = 35-36 minutes,

and AE with BHT at around t_R = 57 minutes due to their chromatographic behavior which are quite comparable in the considered analytical conditions.

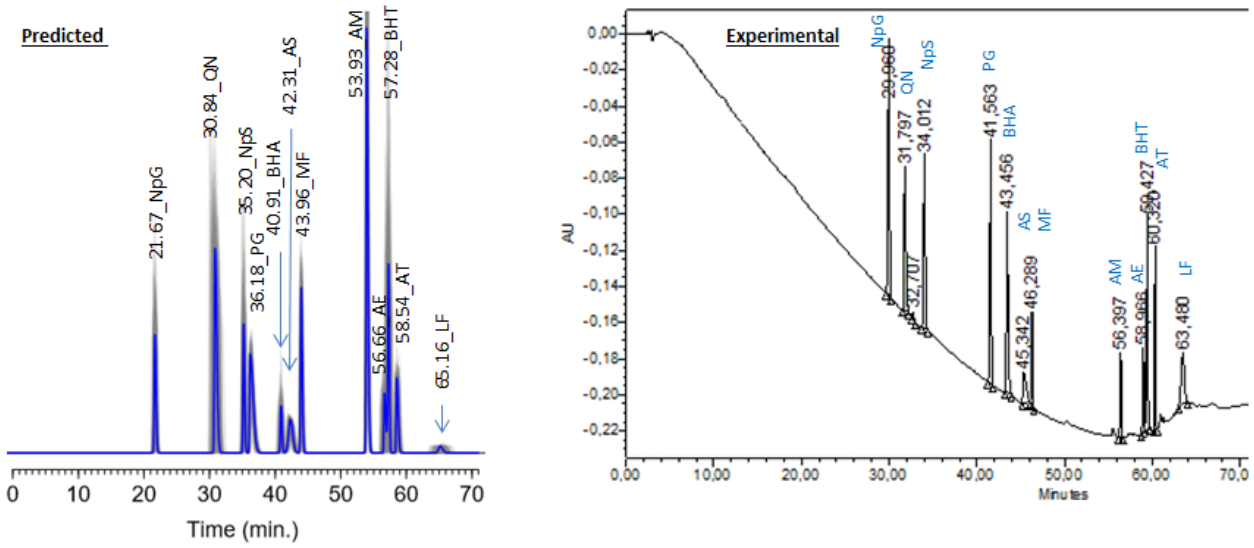

Fig. 7.a *Predicted and experimental chromatograms for the screening method of the 12 analytes*

Experimental conditions: Column: SunFire C18, 250 x 4.6 mm, dp: 5 µm; Flow: 1 mL min^{-1}; T°: 35°C; λ = 230 nm; Gradient elution with methanol and 10 mM ammonium acetate buffer pH 5.65:

Time (min)	Methanol	Buffer	Elution phase type
00.0	5	95	Gradient
60.0	95	5	
70.0	95	5	Isocratic plate
71.0	5	95	Re-equilibration
100.0	5	95	

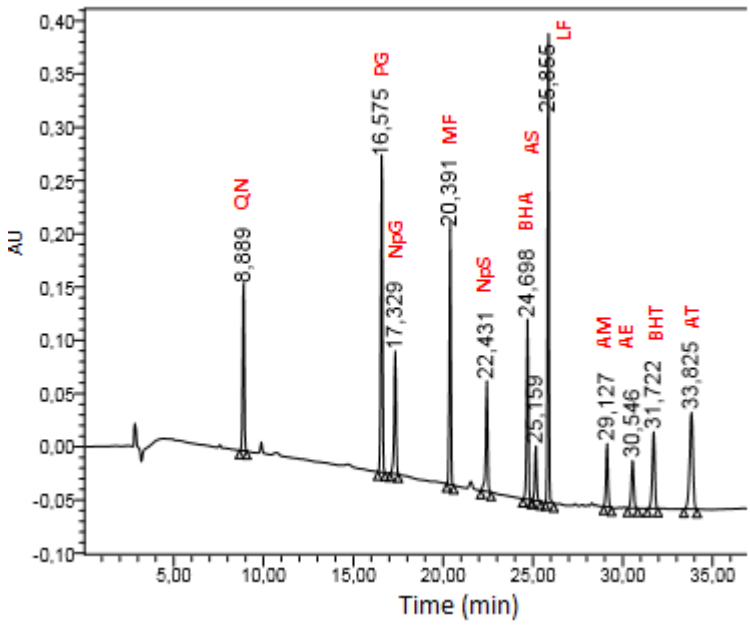

Fig. 7.b *Observed chromatogram by the second screening method for the analysis of the 12 analytes*

Experimental conditions: Column: SunFire C18, 250 x 4.6 mm, dp: 5 µm; Flow: 1 mL min^{-1}; T°: 35°C; λ = 230 nm; Gradient elution with methanol and 10 mM ammonium formate buffer pH 2.5:

Time (min)	Methanol	Buffer	Elution phase type
00.0	10	90	Gradient
25.0	90	10	
35.0	90	10	Isocratic plate
36.0	10	90	Re-equilibration
65.0	10	90	

Note that the predicted and experimental chromatograms are quite comparable in terms of peaks elution order and retention times which changed at around ± 2 min of the predicted t_R in general except for QN, AS and PG that changed at + 7 min, + 5 min and + 3 min respectively due to the change of the intrinsic nature of the SunFire column used in the experimentation vs. the original database created with a XBridge column. Moreover, we calculated the relationship between the predicted and observed t_R for the screening method, and we found R^2 = 0.9697 which indicates the fitness of the method in giving good chromatographic results at around 97% of success on a SunFire column. On the other hand, the sub-screening method tested on a the same SunFire column gave very satisfactory results in terms of peak resolutions where all peaks are perfectly separated and identifiable (cf. **Fig. 8**). The change of elution order in this method is also caused by the intrinsic nature of the SunFire column used during the experimentation.

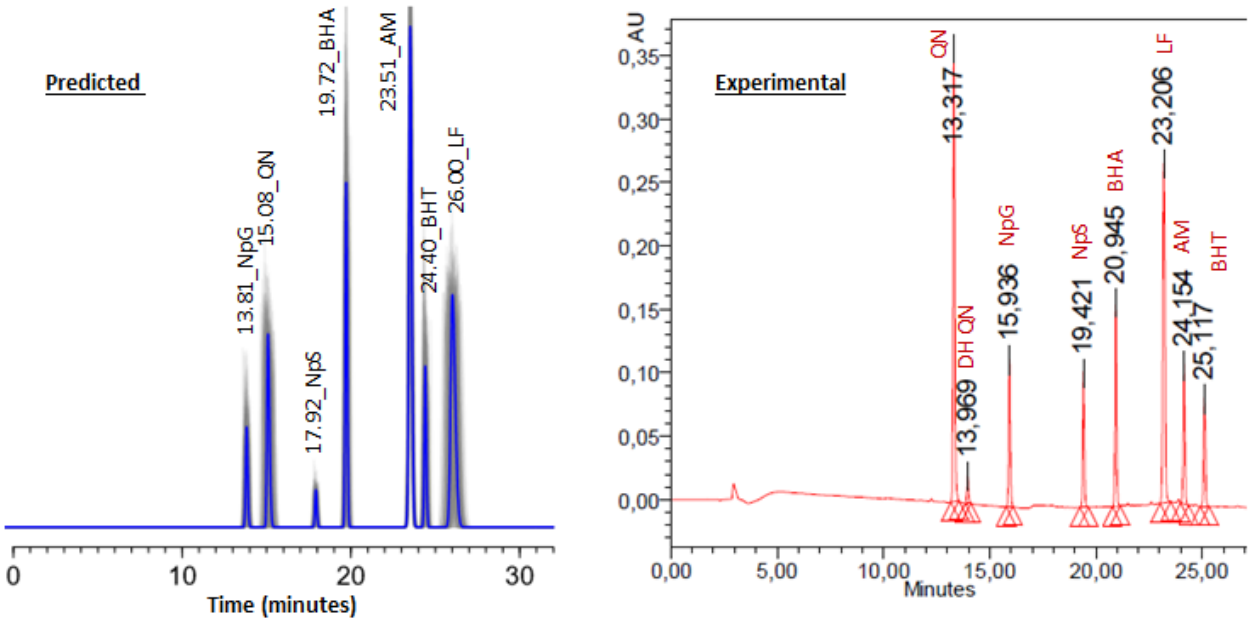

Fig. 8 *Predicted and experimental chromatograms for the screening method of QN; AM / LF and 4 excipients (BHA, BHT, NpG, NpS) in liquid dosage forms.*

Experimental conditions: Column: SunFire C18, 250 x 4.6 mm, dp: 5µm; Flow: 1 mL min^{-1}; T°: 25°C; λ = 230 nm; Gradient elution with methanol and 10 mM ammonium formate buffer pH 4.3:

Time (min)	Methanol	Buffer	Elution phase type
00.0	5	95	Gradient
20.0	95	5	
30.0	95	5	Isocratic plate
31.0	5	95	Re-equilibration
60.0	5	95	

On the other hand, from the modelled retention times of different compounds with respect to the DoE factors (i.e. TG, T°, and pH) we deducted a sub-screening method applicable to the analysis of QN; AE; AM and AS in injectable forms; and the same method is applicable to the screening of QN, and AM / LF in tablet formulations, as well as for MF; PG and AT in tablet forms too at pH 2.5, TG: 20 minutes and temperature at 25°C whose chromatograms are illustrated in **Fig. 10** after geometric transfer of the method on shorter analytical columns.

4.3 Geometric transfer of the screening method

To reduce the analysis time, we performed geometric transfer of the methods from long to short analytical columns; and the transferred analytical conditions were obtained thanks to the HPLC Calculator v3.0 freeware [11]. The **Fig. 9** illustrates an example of chromatograms

obtained on short columns; and from these results, one can notice an important decrease of the run time to 41.5 min on a 150 x 4.6 mm, (dp: 5µm) column, and to 23.3 min on a 100 x 4.6 mm, (dp: 3.5 µm) compared to 65 min on a 250 x 4.6 mm, (5 µm) column used during the method development. In order to further reduce the analysis time of the screening method, a shortened gradient profile described in **Table 4** was tested and gave interesting results presented in the same table, but we suggest to consider this method as a larger sub-screening method capable to screen 10 analytes simultaneously within 19 minutes. We recommend to use this method on samples not containing Mefloquine and Nipasol together or Arteether and Lumefantrine together because the two couples of molecules co-elute at around 7.3 minutes and 9.3 minutes of the t_R (min.) respectively. All other analytes are perfectly separated with this method. Thus far, the screening method can further be transferred to the shortest conventional column and therefore allowing to screen individually the four studied sub-groups within 11 minutes as illustrated in **Fig. 10**.

Hence, the transfer of these methods to different analytical columns allows the analysts to have a wide range of choice among columns available in the laboratory, and therefore apply the appropriate analytical conditions during the analysis of suspected counterfeit samples.

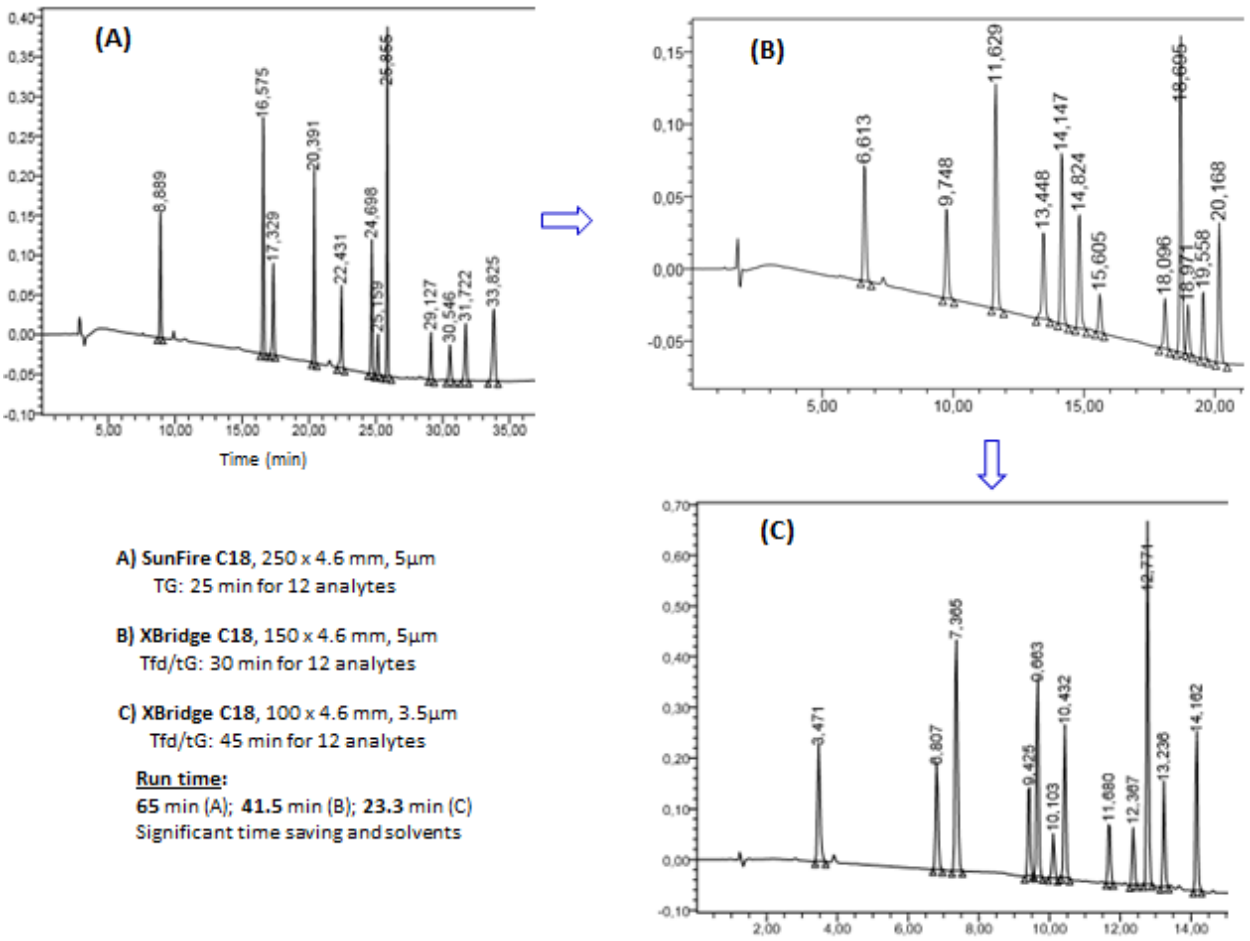

Fig. 9 *Geometric transfer of the screening method from SunFire C18, 250 x 4.6 mm, (5 µm, dp) to XBridge C18, 150 x 4.6 mm, (5 µm, dp) and C18, 100 x 4.6 mm, (3.5 µm, dp)*

Experimental conditions:

(A) Idem to **Fig. 7.b**

(B) Column: XBridge C18, 150 x 4.6 mm, dp: 5µm; Flow: 1 mL/min; T°: 35°C; λ = 230 nm; Gradient elution with methanol and 10 mM ammonium formate buffer pH 2.5:

Time (min)	Methanol	Buffer	Elution phase type
00.0	10	90	Gradient
17.5	90	10	
23.5	90	10	Isocratic plate
24.1	10	90	Re-equilibration
41.5	10	90	

(C) Column: XBridge C18, 100 x 4.6 mm, dp: 3.5µm; Flow: 1 mL/min; T°: 35°C; λ = 230 nm; Gradient elution with methanol and 10 mM ammonium formate buffer pH 2.5:

Time (min)	Methanol	Buffer	Elution phase type
00.0	10	90	Gradient
12.1	90	10	
14.9	90	10	Isocratic plate
15.2	10	90	Re-equilibration
23.3	10	90	

Elution order:

Chromatogram (A): QN; PG; NpG; MF; NpS; BHA; AS; LF; AM; AE; BHT; AT
Chromatogram (B): QN; NpG; PG; NpS; MF; BHA; AS; AE; LF; AM; BHT; AT
Chromatogram (C): QN; NpG; PG; NpS; MF; AS; BHA; AE; AM; LF; BHT; AT

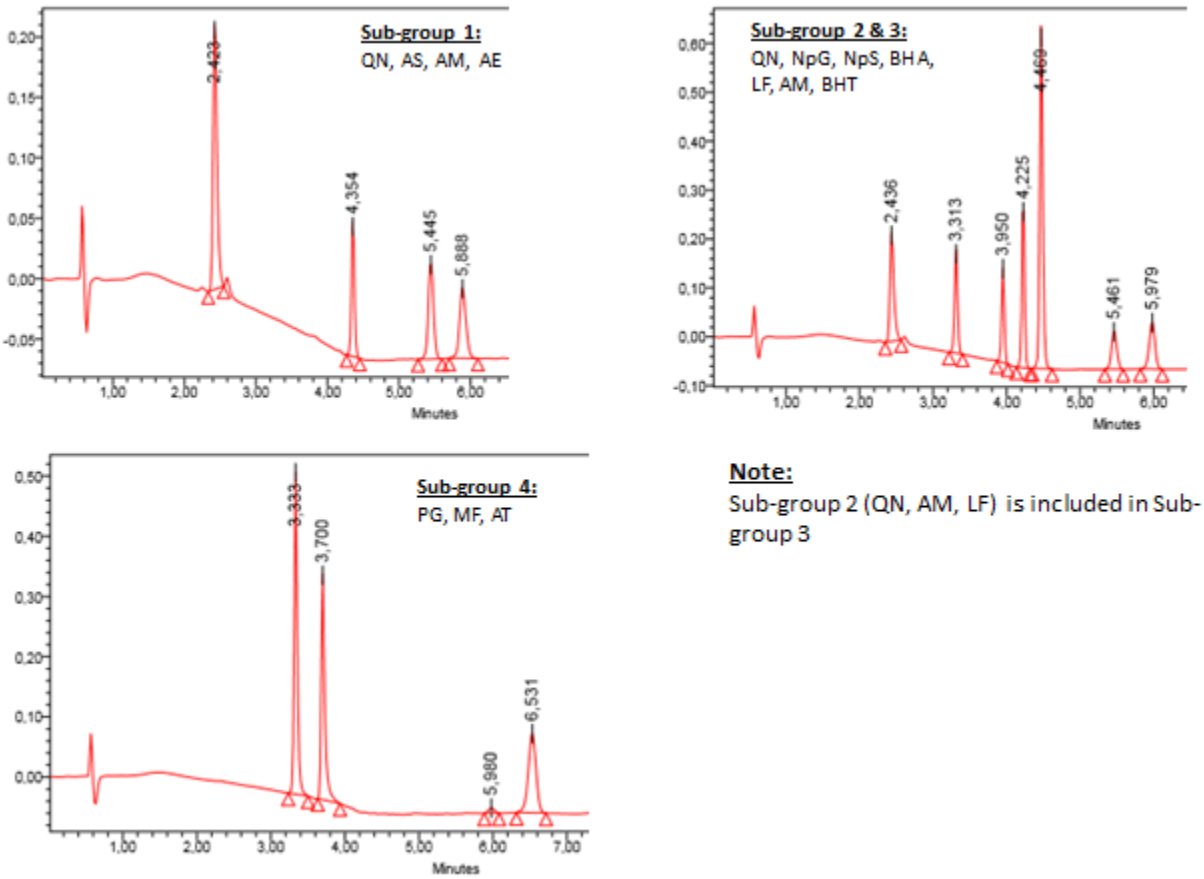

Fig. 10 *Transfer of the second screening method on the shortest conventional analytical column for the screening of the 4 studied sub-group categories of antimalarial medicines*

Experimental conditions: Column: XBridge C18, 50 x 4.6 mm, dp: 5µm; Flow: 1 mL/min; T°: 25°C; λ = 230 nm; Gradient elution with methanol and 10 mM ammonium formate buffer pH 2.5:

Time (min)	Methanol	Buffer	Elution phase type
0.0	5	95	Gradient
3.0	95	5	
5.0	95	5	Isocratic plate
5.2	5	95	Re-equilibration
11.0	5	95	

Elution order:

Sub-group 1 chromatogram: QN; AS; AM; AE
Sub-group 2 chromatogram: QN; LF; AM
Sub-group 3 chromatogram: QN; NpG; NpS; BHA; LF; AM; BHT
Sub-group 4 chromatogram: PG; MF; AT

4.4 Application of the methods on other compounds

As shown in **Table 4**, the screening method was successively tested on various other compounds that can be used by counterfeiters instead of the eight studied antimalarial medicines which are AE, AM, AS, AT, LF, MF, PG and QN with four major preservatives and antioxidants BHA, BHT, NpG, and NpS. Then, the results revealed that the method can easily detect 11 other types of antimalarial medicines not officially used in Rwanda i.e. piperaquine, amodiaquine, chloroquine, cinchonine, sulfadoxine, pyrimethamine, sulfalene, halofantrine, primaquine, artemisinine and dihydroartemisinine; 14 non-steroidal anti-inflammatories and related compounds: diclofenac, caffeine, acetylsalicylic acid, ibuprofen, atenolol, paracetamol, sodium benzoate, dexamethasone, codeine, prednisolone, naproxen, furosemide, methylprednisolone, and diazepam; and 13 antibiotics: amoxicillin, ampicillin, trimethoprim, levofloxacin, chloramphenicol, phenoxymethylpenicillin, clindamycin, sulbactam, tazobactam, tetracycline, cefotaxime, ceftriazone, and ciprofloxacin. This application of the method on other molecules is very useful in the sense of demonstrating the high capability of HPLC technique in detecting a wide range of other molecules that can be used in counterfeiting.

Table 4

Peak separation by the shortened gradient profile on XBridge C18, 100 x 4.6 mm, dp: 3.5µm; Flow: 1 mL/min; T°: 35°C; λ = 230 nm; Gradient elution with methanol and 10 mM ammonium formate buffer pH 2.5:

Time (min)	Methanol	Buffer	Elution phase type
0.0	10	90	Gradient
7.9	90	10	
10.7	90	10	Isocratic plate
11.0	10	90	Re-equilibration
19.0	10	90	

	A. Targeted 12 analytes (Rwanda)			***B. Other antimalarials***	
#	**Molecule**	**t_R (min)**	**#**	**Molecule**	**t_R (min)**
1	Quinine	3.20	1	Piperaquine	1.90
2	Nipagin	5.70	2	Cinchonine	2.43
3	Proguanil	5.94	3	Chloroquine	3.32
4	Mefloquine	7.34	4	Amodiaquine	3.70
5	Nipasol	7.37	5	Sulfalene	4.17
6	Artesunate	7.74	6	Sulfadoxine	4.48
7	BHA	8.00	7	Primaquine	4.94
8	Artemether	8.83	8	Pyrimethamine	5.07
9	Arteether	9.21	9	Dihydroartemisinin	7.40
10	Lumefantrine	9.30	10	Artemisinin	7.56
11	BHT	9.74	11	Halofantrine	8.90
12	Atovaquone	10.43	-	-	-
	C. NSAIs and related molecules			**D. Antibiotics**	
#	**Molecule**	**t_R (min)**	**#**	**Molecule**	**t_R (min)**
1	Atenolol	2.07	1	Sulbactam	2.18
2	*Metamizole**	2.17	2	Ceftriaxone	2.23
3	Codeine	2.53	3	Tazobactam	2.63
4	Paracetamol	2.70	4	Amoxicillin	2.71
5	Caffeine	3.61	5	Trimethoprim	3.43
6	Aspirin	4.79	6	Cefotaxime	3.86
7	odium benzoate	5.37	7	Tetracycline	3.88
8	*(Unidentified)***	6.04	8	Ampicillin	4.00
9	Furosemide	6.28	9	Levofloxacin	4.11
10	Prednisolone	6.57	10	Ciprofloxacin	4.34
11	Dexamethasone	7.00	11	Chloramphenicol	5.40
12	Methylprednisolone	7.13	12	Clindamycin	5.54
13	Diazepam	7.33	13	Penicillin V	6.87
14	Naproxen	7.60	-	-	-
15	Ibuprofen	8.54	-	-	-
16	Diclofenac	8.63	-	-	-

Note: * Metamizole was poorly detected; its peak was very negligible nearer to the baseline noise.

** A very significant peak was detected, but not expected. We assume that it is Ibuprofen photodegradant

Thus, after noticing how much this screening method had sharply detected all those molecules, there is a guarantee that several other compounds that can be used by counterfeiters will be easily detected too, and therefore be discarded from the supply chain.

5. CONCLUSION

An earlier developed DoE-DS database was exploited during the optimization of: (i) an overall screening method for the analysis of eight antimalarial medicines officially used in Rwanda with four major excipients (i.e. arteether, artemether, artesunate, atovaquone, lumefantrine, mefloquine, proguanil, and quinine with BHA, BHT, nipagin and nipasol); (ii) a sub-screening method for the analysis of quinine, artemether and lumefantrine in liquid dosage forms containing the four excipients; and (iv) a generic sub-screening method for the analysis of quinine, arteether, artemether, and artesunate in injectable forms; mefloquine, atovaquone and proguanil in solid dosage forms; quinine, artemether and lumefantrine in tablet forms. These methods are mainly useful in the analysis of suspected counterfeit antimalarial medicines containing or not any of the four studied preservatives and antioxidants.

Then, to reduce the analysis time and increase the sample analysis throughput, the methods were geometrically transferred to shorter analytical columns, and another advantage is the reduction of the mobile consumption and the inherent liquid wastes.

Moreover, the screening method was applied in testing other types of classical compounds in the range of other antimalarial medicines not officially used in Rwanda, non-steroidal anti-inflammatories and related compounds, and antibiotics in order to assess whether the method has the capacity of detecting other components that could be used by counterfeiters, and the method was found very effective.

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IV.3 SECTION 3 - Simple LC isocratic Methods development, Validation, and Application in the Analysis of poor quality antimalarial medicines

American Journal of Analytical Chemistry, 2017, 8, 582-603
http://www.scirp.org/Journal/PaperInformation.aspx?PaperID=79155

IV.3.1 Preamble to Article 3

Very often, in developing countries, we find LC equipment with binary pumps or other ones for which the gradient mode cannot be used. It is therefore important to have simple analytical methods that are applicable to both systems in order to increase the pool of utilization either on one system or another. This is particularly relevant for the analysis of vital and essential medicines such as the antimalarials that are highly targeted by counterfeiters and that have to be analyzed more rapidly. So in this section we present a methodological approach for the development of simple LC methods for routine analysis of antimalarial medicines widely marketed in generic forms, and which are most likely to be counterfeit or substandard. To fulfill the needs for such methods and context, some technical and economical criteria were obvious namely their simplicity, short analysis time, low mobile phase consumption, adaptability to LC systems equipped with simple or gradient pumps, large flexibility to tolerable changes on the mobile phase organic and aqueous proportions, column dimensions, flow rate, etc. without need of revalidation, and of course transferability.

We have also considered the use of methanol as organic modifier that is available compared to acetonitrile which is expensive (almost three times more than the cost of methanol), and the use of ammonium formate buffer that is volatile without risk of precipitation in the LC system.

The following antimalarial medicines were included in this study:

- Quinine with major cinchona alkaloids (cinchonidine and dihydroquinine) in tablet forms;
- Quinine resorcin with major cinchona alkaloids (cinchonine, cinchonidine, quinidine, and dihydroquinine) with resorcinol in injectable solutions;
- Artemether / lumefantrine tablet forms; and

- Arteether, artemether, artesunate in injectable forms.

The method for analysis of artesunate powder for injection was successfully validated and applied on real samples, and different other samples of artemether / lumefantrine tablets from different places in Rwanda, DRC and Benin were also analyzed. Bad results were obtained on three samples of artemether / lumefantrine tablets that were found out-of-specifications, and very alarming results were found on fake quinine tablets and fake Coartem (artemether / lumefantrine) that did not contain any of the declared active ingredients.

IV.3.2 Article 3

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Simple LC Isocratic Methods Development, Validation, and Application in the Analysis of Poor Quality Antimalarial Medicines

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ABSTRACT

Liquid chromatographic methods in isocratic mode for the analysis of poor quality medicines are privileged due to their simplicity and facility in methods development. They are generally fast; do not need to be re-equilibrated between sample injections; have larger flexibility with acceptable changes on different column dimensions; and are applicable to LC systems equipped with simple or high developed pumps. In this study, we focused on developing simple isocratic methods using classical mobile phase composed by methanol and ammonium formate buffer for the analysis of most common antimalarial medicines marketed in malaria endemic countries and susceptible of being counterfeit/falsified, substandard and degraded.

The selected medicines were quinine and related cinchona alkaloids in tablets and injectable forms; artemether/lumefantrine tablets; and artemisinin compounds (arteether, artemether, and artesunate) in injectable forms. The current methods were developed thanks to simple methodological approach consisting in sequential isocratic runs through adjustment or adaptation of existing methods to obtain optimal analytical conditions without complex

design of experiments that might be long and costly. Then, the new methods presented shorter analysis time; allowed increase of sample analysis throughput; and obviously consumed little mobile phase solvents on classical analytical columns: 50 – 250 mm of length (L), 4.6 mm of internal diameter (I.D.), and 3.5 - 5.0 μm of particle size (dp).

1. INTRODUCTION

Malaria is a life-threatening disease caused by Plasmodium parasites transmitted to people through the bites of infected female Anopheles mosquitoes (called malaria vectors). It is usually found in tropical and subtropical climates where the parasites live [1] [2]. According to the latest estimates of the World Health Organization (WHO), released in end of 2016, there were 212 million cases of malaria in 2015 and 429,000 deaths. The WHO African Region is the most affected by that disease and its consequences [1]. Thus far, the prevention of malaria involves among others the use of "insecticide-treated mosquito nets" and "indoor residual spraying" as effective vector control mechanisms, and the use of "antimalarial medicines" in disease treatment or chemoprophylaxis. Concerning medicines, they should always meet their quality specifications in order to give guarantee on their safety and efficacy during their shelf lives. Otherwise, any failure to the required quality standards may lead to serious public health concerns such as failure in disease treatment, development of drug resistance, increase of morbidity and mortality, etc.

Indeed, poor quality medicines constitute a harmful threat to the public health worldwide, particularly in under-resourced countries [3] [4] [5]. Newton et al. [3] distinguished three categories of poor quality medicines: (i) Counterfeit/falsified medicines which are illicit products maliciously produced and distributed; (ii) Substandard also called out-of-specification "OOS" products which are genuine products generally produced in poor manufacturing conditions; and (iii) Degraded medicines which are products improperly stored, and spoiled. Hence, there is need to develop fast, effective, simple and transferable analytical methods to drug quality control laboratories in developing countries, and therefore reinforce their capacity in detecting and fighting against the spread of those harmful products.
In this context, we have developed simple isocratic methods for the analysis of curative antimalarial medicines most used in Rwanda i.e. artemether/lumefantrine, artesunate, and quinine with related other compounds that should be associated with them in case of counterfeiting, substandard, or degradation. This is the case for example of quinine and

related cinchona alkaloids such as cinchonine, cinchonidine, quinidine, and dihydroquinine, together with resorcinol most found in quinine resorcin formulations, etc. Moreover, we added arteether to the group of artemisinin derivatives that should be easily interchanged with artemether and artesunate due to their closer chemical structure similarity (see **Figure 1**).

The technique of Liquid Chromatography (LC) coupled with UV detector or DAD was selected as gold standard for chemical separation, quantification and identification of simple and complex samples. This technique is highly used in pharmaceutical analysis, very sensitive and accurate; it gives results in relatively good-time, generally within approximately 10 and 15 minutes for isocratic methods, and within less than 60 minutes for gradient methods; and has a great capacity of automation [5] [6].

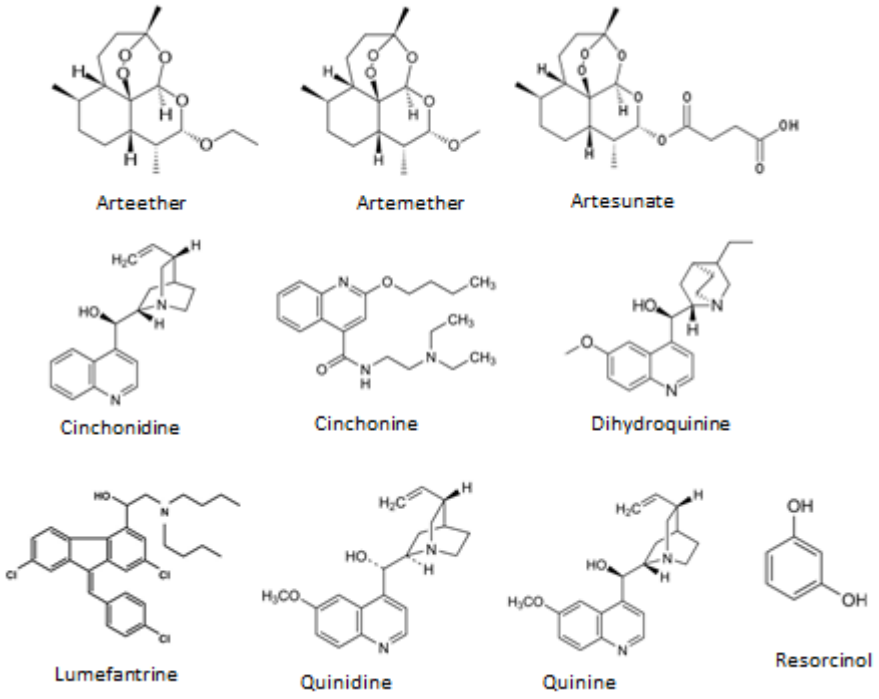


Figure 1 *Molecular structures of the studied analytes*

The targeted methods were privileged for their simplicity, shorter analysis time, and higher analysis sample throughput; they were developed thanks to the adjustment (fine-tuning) or adaptation of other existing methods in fewer experimental runs without passing through design of experiments that are information fullness however with high cost and relatively long time consuming. Then, after optimizing the new methods, they must be validated according to

the International Conference on Harmonization “ICH-Q2(R1)” guidelines [7], and we used the total error strategy based on random and systematic errors corresponding to the precision + trueness, and using accuracy profiles as decision tool on the fitness of the methods for their intended use within given acceptable limits [8] [9] [10] [11].

Finally, in the frame of reducing the analysis time, we transferred the methods from long to shorter analytical columns in the classical range of 250 mm and 50 mm of length (L) × 4.6 mm of internal diameter (ID), and 5.0 to 3.5 μm of the particle size (dp) before use in routine analysis of different medicines especially when detecting counterfeit formulations.

2. MATERIALS AND METHODS

2.1 Materials

Arteether (100%), Artemether (100%), Artesunate (> 97%), and Lumefantrine (100%) were purchased from Sensa Pharm (Lanchester, UK), and quinine sulfate (99.0%) from Fagron N.V. (Waregem, Belgium), quinine dihydrochloride (100.8%) from Molekula Limited (Dorset, UK), Hydrochloric acid (37%) and ammonium formate (98.1%) from VWR International BVBA (Leuven, Belgium), Methanol LC grade from Avantor Performance Materials B.V. (Deventer, The Netherlands), the Ultrapure Water was produced with a Milli-Q Plus 185 water purification system (Millipore, Billerica, MA, USA), and various samples of quinine tablets (300 mg), quinine for injection (300 mg/mL and 600 mg/mL) as labeled on the primary packaging, artemether/lumefantrine tablets (20 mg / 120 mg and 80 mg / 480 mg), and artesunate powder for injection (60 mg) were randomly collected from Rwanda, Democratic Republic of Congo, and Benin.

2.2 Methods development strategy and validation

Methods in isocratic mode were developed by simple systematic approach, and by adaptation or adjustment of other methods on similar compounds. These methodologies have an advantage of reducing the number of experiments to be carried out without going through complex design of experiments (DoEs). The strategy of simple systematic approach was based on sequential isocratic runs and optimization of the most promising results, while the methods adaptation or adjustment was based on other methods found in literature (scientific

publications) on the same analytes, and tested with adequate adaptations or adjustments to have the desired chromatographic conditions using our preferred mobile phase composed by methanol and ammonium formate buffer.

2.3 Instrumental and software

2.3.1 LC equipments

The methods were developed in Belgium on a Waters 2695 Alliance HPLC Separation Module coupled to Waters 2996 photodiode array (PDA) detector from Waters Corporation (Milford, MA, USA) piloted with Empower 2.0 software (Waters Corporation). Different chromatographic columns packed with C18 or C8 stationary phases, in different dimensions (L: 50 mm to 250 mm, ID: 4.6 mm; dp: 3.5 µm and 5 µm) were used during the methods development and geometric transfer as described in the experimental part.

2.3.2 Software

Empower 2.0 software (Waters Corporation, MA, USA) for Windows was used to control the Waters Alliance HPLC system, to record the signals from the detector and interpret the generated chromatograms. Then, different optimum levels for geometric transfer from columns to others were calculated using HPLC Calculator v3.0 developed by Guillarme et al. [12], and the accuracy profiles as well as the statistical calculations including the validation results and uncertainty estimates were obtained thanks to e-noval® V3.0 software (Arlenda, Belgium).

2.4 Preparation of sample solutions

2.4.1 Sample solutions for method development for the analysis of arteether, artemether, artesunate and quinine in different injectable formulations

In the first step, approximately 10.0 mg of quinine base from quinine dihydrochloride chemical reference substance (CRS) were dissolved with methanol in 100.0 mL volumetric flask; and in the second step, this solution was used to dissolve and dilute approximately 10.0 mg of each of the arteether, artemether and artesunate reference substances weighed in a 2.0

mL volumetric flask. This was the final working solution annotated “Solution Q3A” to be injected in the chromatographic system. It contained approx. 100 μg mL^{-1} of quinine, and 5000 μg mL^{-1} of the three artemisinin derivate compounds.

2.4.2. Sample solutions for method adaptation for the analysis of quinine, resorcinol, quinidine, dihydroquinine and other cinchona alkaloids in tablets and injectable formulations

The working sample solutions were prepared from two real medicines containing the studied analytes (i.e. quinine, dihydroquinine, quinidine, cinchonine, cinchonidine, and resorcinol) manufactured by Sanofi-Winthrop Pharma (Senegal) and Wintac Ltd. (India).

Each sample solution was diluted with purified water to have a final working solution containing approximately 100 μg mL^{-1} of quinine alkaloid which is the main active ingredient, and the concentrations of other cinchona alkaloids together with resorcinol were subsequently reduced according to the product formulation as stated on the labeled composition. For example, one sample of ampoule 4 mL was stated to contain per mL: 96.10 mg of quinine resorcinol dihydrochloride, 2.55 mg of quinidine resorcinol dihydrochloride, 0.68 mg of cinchonine resorcinol dihydrochloride, 0.67 mg of cinchonidine resorcinol dihydrochloride, and water for injection BP q.s. Then, to have final working solutions containing approx. 100 μg mL^{-1} of quinine resorcinol dihydrochloride, 520 μL of quinine resorcin injection sample were diluted in 500 mL volumetric flasks before they were injected in the chromatographic system for analysis. Note that the content of resorcinol was not mentioned on both samples, but the product was well identified as shown later in results. Then, to identify all six analytes, we injected separately in the LC system single solutions of quinine reference substance (100 μg mL^{-1} in purified water) containing dihydroquinine (<10%), cinchonine CRS (50 μg mL^{-1} in purified water), cinchonidine CRS (50 μg mL^{-1} in purified water), quinidine CRS (50 μg mL^{-1} in purified water), and resorcinol CRS (50 μg mL^{-1} in purified water too).

2.4.3. Sample solutions for method adjustment for the analysis of artemether / lumefantrine in tablet formulations

Reference to the USP, International and European pharmacopoeias allowable adjustments in chromatographic systems [13] [14] [15], our previously validated method for the analysis of artemether/lumefantrine in tablets forms [16] was adjusted in order to reduce the analysis time and increase the sample analysis throughput. The following solutions were prepared:

Standard solutions:

Dissolve accurately weighed quantities of artemether and lumefantrine reference substances in appropriate volumetric flask with acidified methanol by phosphoric acid (0.2% phosphoric acid in methanol, w/v) to obtain 200 and 1200 μg mL^{-1} of both analytes respectively. Prepare two independent standard solutions for system suitability testing and analysis.

Sample solutions:

Different samples randomly collected using blind sampling techniques from Rwanda (n = 13), D.R. Congo (n = 9), and Benin (n = 1 suspected counterfeit) were prepared for analysis in the frame of quality assessment of artemisinin-based combination therapy (ACT) medicines marketed in the three countries.

The sample solutions were prepared by weighing and powdering 20 tablets; and transferring a quantity of each sample powder containing approximately 20 mg of artemether and 120 mg of lumefantrine accurately weighed in 100.0 mL volumetric flask, and dissolving the sample with the acidified methanolic solution (phosphoric acid 0.2%, w/v) used in the preparation of artemether/lumefantrine standard solutions by mechanical shaking on vortex during 1 minute, and dilution to volume with the same solvent to obtain approx. 200 and 1200 μg mL^{-1} of both analytes respectively. Prepare three independent sample solutions per batch, and filter each solution through 0.45 μm filter before they are injected in the chromatographic system.

2.4.4. Sample solutions for method validation and routine analysis for artesunate powder for injection

a) Sample solutions for method validation

They consisted of three concentration levels for calibration standards (CS), and five concentration levels for validation standards (VS). The required solutions were prepared as described in section 3.2.

b) Sample solutions for routine analysis

The samples of artesunate powder for injection were prepared by dissolving with methanol LC grade approximately 10.0 mg of the sample in 2.0 mL volumetric flasks and completing to volume with the same solvent to obtain around 5000 μg mL^{-1} final solutions against artesunate reference substance prepared at the same concentration level with the same solvent. Three independent sample solutions were prepared, and two independent reference solutions for system suitability testing and sample analysis.

3. EXPERIMENTATION

3.1. Simple isocratic methods development by adaptation or adjustment and sequential isocratic runs with fine-tuning

Simple isocratic methods for the analysis of (i) quinine and artemisinin derivatives (arteether, artemether, and artesunate), (ii) quinine resorcin and major cinchona alkaloids (cinchonine, cinchonidine, quinidine, and dihydroquinine), and (iii) artemether / lumefantrine in different pharmaceutical forms were developed through sequential isocratic runs with optimization (fine-tuning), and by simple adaptation or adjustment of other methods in order to reduce the analysis time, improve the sample treatments conditions, and use of our preferred mobile phase composed by methanol and ammonium formate buffer which is not expensive and that can be transferred to LC/MS for advanced analysis whenever needed for the identification of unknown compounds. Moreover, isocratic methods have an advantage of flexibility to tolerable changes with column dimensions, flow rate of the mobile phase, organic modifier proportion, etc. that can be done without need of the methods revalidation [13] [14] [15].

3.1.1. Development of a generic method for the analysis of quinine, arteether, artemether and artesunate in injectable formulations

The development of a generic isocratic method for the analysis of quinine, arteether, artemether and artesunate was done through a simplified systematic approach based on sequential isocratic runs with optimization (fine-tuning) described in **Table 1**, using classical analytical columns and mobile phase composed by methanol and ammonium formate buffer.

The first three experiments helped to know the trend of peak separation of the four analytes either at higher or lower proportion of the organic modifier, and therefore have an idea on which parameter to make changes in the optimization phase. For example, if the best separation of the analytes is at the lower level of methanol (i.e. at Test 1: 25/75% MeOH/Buffer, v/v), one can plan for two additional tests at 25% ± 10% of methanol (i.e. Test 4: 15/85% and Test 5: 35/65% MeOH/Buffer, v/v), and then continue the adjustment of the method with the flow rate changes at 0.7 mL min^{-1} or 1.3 mL min^{-1} if needed (Test 6), temperature changes at 35˚C (Test 7), or columns dimensions (Test 8: L = 50 - 250 mm; ID: 4.6 mm; dp: 3.5 or 5 μm) in order to have the desired chromatographic separation within ≤ 10 minutes of run time generally preferred for isocratic methods.

Table 1

Simplified sequential isocratic experiments

Factor	**Experiments**			
	Test (1)	**Test (2)**	**Test (3)**	**Optimization (± 5 tests)**
Mobile phase :	< 50%	50:50%	> 50%	About five more tests are added: For example at the Flow of 1.0 ± 0.3 mL min^{-1}. (0.7 mL min^{-1} and 1.3 mL min^{-1}); 25 or 75 ± 10%, v/v of organic modifier (15:85%, or 35:65%; 65:35% or 85:15%) v/v, column dimensions, or column temperature adaptations, etc.
Methanol	25%	50%	75%	
NH_4 formate buffer (*)	75%	50%	25%	
Flow rate (mL min^{-1})	1.0	1.0	1.0	
Column temperature (°C)	25	25	25	
Injection volume (μL)	10	10	10	
Detection wavelength (nm)	210	210	210	

(*): The aqueous mobile phase is 10 mM ammonium formate pH 2.8

3.1.2. Development of isocratic method for the analysis of quinine, resorcinol, dihydroquinine and major cinchona alkaloids

The analysis of quinine in different pharmaceutical forms is generally done with other cinchona alkaloids especially dihydroquinine that should not be more than 10% of content, cinchonidine not more than 5%, and any other related substance such as cinchonine, quinidine, etc. at not more than 2.5% calculated by the area percentage method to quinine peak area [17].

Reference to other existing methods covering almost the same analytes [18] [19], we optimized a rapid isocratic method for the analysis of quinine, resorcinol, cinchonine, cinchonidine, quinidine, and dihydroquinine by adapting these methods to our analytes and changing the earlier mobile phase to methanol and ammonium formate buffer. Resorcinol was added to the list of the studied analytes, as being one of the key ingredients in quinine resorcin injection solutions widely used in different malaria endemic countries.

The method was optimized on a Zorbax SB-C8 (dp 3.5 μm) column, (150 mm × 4.6 mm ID) maintained at 35˚C, applying as mobile phase an isocratic mixture of methanol and 10 mM ammonium formate buffer (adjusted to pH 2.8 with formic acid or 6 N hydrochloric acid) (40:60, v/v) for the analysis of quinine tablets containing generally quinine sulfate, dihydroquinine, and cinchonidine; and (30:70, v/v) for the analysis of quinine resorcin injection containing the six analytes (quinine, resorcinol, dihydroquinine, cinchonine, cinchonidine, and quinidine), at a flow rate of 1 mL min^{-1}. The sample solutions were thermostated at 15˚C, introduced in the separation system at 10 μL injection volumes, and monitored at 230 nm.

3.1.3. Adjustment of the method for analysis of artemether / lumefantrine in tablet formulations

Our former analytical method for the analysis of artemether/lumefantrine in tablet formulations [16] was readjusted to improve the analysis time (run time), and sample treatment by increasing the capacity of methanol in dissolving lumefantrine. Hence, to improve the analysis time, we slightly increased the proportion of methanol in the mobile phase and the run time reduced; then, to increase the capacity of methanol in dissolving

lumefantrine, we slightly increased the acidity power by using methanol acidified with phosphoric acid 0.2% (w/v) instead of 0.1% (w/v) previously used.

The method was adjusted on a Zorbax 80Å Extend-C18, 100 mm × 4.6 mm (ID), (dp: 3.5 µm) chromatographic column at a flow rate of 0.7 mL min^{-1} of methanol and 10 mM ammonium formate buffer pH 2.8 (85:15%, v/v) maintained at 25˚C, injecting 6 µL of the sample and reference solutions, and recording the chromatographic data at 210 nm. The dissolution of lumefantrine was improved by slight increases of the phosphoric acid in methanol from the original content of 0.1% to 0.2% (w/v) that is enough to dissolve the targeted analyte by hand shaking or vortex easily.

3.2. Validation of the method for analysis of artesunate powder for injection

The generic isocratic method for the analysis of quinine and artemisinin derivatives was validated for specific analysis of artesunate powder for injection. The calibration and validation standard solutions were prepared by dissolving artesunate CRS in methanol in order to have the following solutions.

Calibration standards :

Level 1 (60%): 3000 µg mL^{-1} of artesunate (approx. 15.0 mg in 5.0 mL vol. flask);
Level 3 (100%): 5000 µg mL^{-1} of artesunate (approx. 10.0 mg in 2.0 mL vol. flask);
Level 5 (140%): 7000 µg mL^{-1} of artesunate (approx. 14.0 mg in 2.0 mL vol. flask).

Validation standards :

The validation standards (VS) were prepared with artesunate CRS and methanol as solvent. There is no sample matrix as the studied product is a pure raw material without any excipient, and the concentration level is higher due to the weak absorption of the UV light by artesunate. The VS were prepared to have five different concentration levels, three series per day, during three validation days.

Level 1 (60%): 3000 µg mL^{-1} of artesunate (approx. 15.0 mg in 5.0 mL vol. flask);
Level 2 (80%): 4000 µg mL^{-1} of artesunate (approx. 20.0 mg in 5.0 mL vol. flask);

Level 3 (100%): 5000 µg mL^{-1} of artesunate (approx. 10.0 mg in 2.0 mL vol. flask);
Level 4 (120%): 6000 µg mL^{-1} of artesunate (approx. 12.0 mg in 2.0 mL vol. flask);
Level 5 (140%): 7000 µg mL^{-1} of artesunate (approx. 14.0 mg in 2.0 mL vol. flask).

To sum up, three independent solutions (n = 3) were prepared per each concentration level (c = 3 for CS, c = 5 for VS), and all these preparations were repeated for three days corresponding to three series (s = 3). The method was validated on a C18, 150 × 4.6 mm, dp: 5 µm column in order to allow covering the widest range of classical HPLC columns' sizes and allowable changes.

Then, for routine analyses, two independent reference solutions for system suitability testing and sample analysis were prepared at 5000 µg mL^{-1} i.e. Level 3 (100%) against three independent sample solutions per batch was prepared at the same concentration level.

3.3. Sampling and application of the methods on real samples

Seventeen sampling sites mapped in **Figure 2** were defined for collecting different antimalarial medicines found on the list of national essential medicines for Rwanda [20] [21] [22]. The targeted sampling points were hospitals and pharmacies in public and private sectors, and tentatively illicit vendors if available, located in strategic cities, suburbs, and Rwanda's borders. The sampling sites were selected on the basis of areas known to have intensive commercial activities, border areas with other countries (Burundi in South, Tanzania in East, Uganda in North, and D.R. Congo in West) where the risk of illegal trade of goods including medicines is relatively higher and therefore the possibility of entry of poor quality medicines especially counterfeit/falsified and substandard. All samples were selected randomly by considering batch numbers, manufacturers, strength, and dosage forms (tablets, capsules, injectables, etc.).

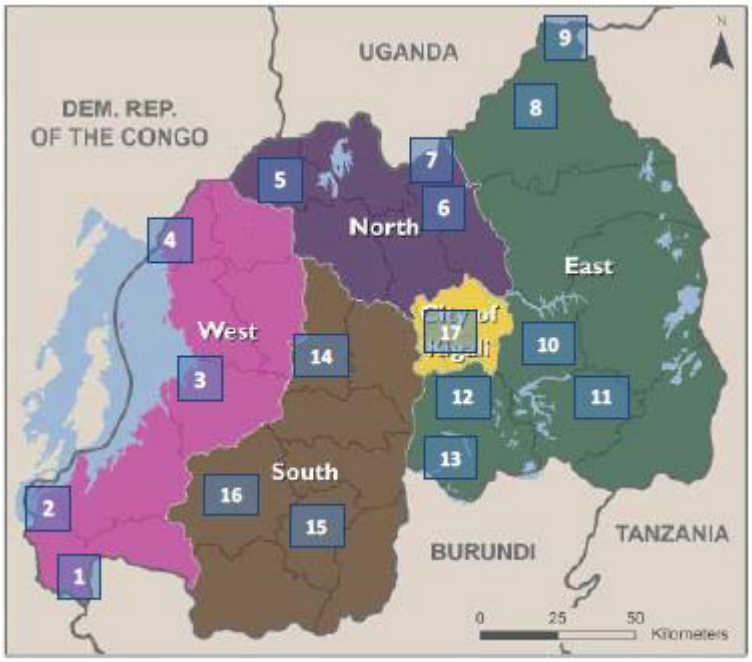


Figure 2 *Strategic sampling sites on the map of Rwanda (**Western Province:** (1) Bugarama, (2) Kamembe, (3) Karongi/Kibuye, (4) Rubavu/Gisenyi; **Northern Province:** (5) Musanze/Ruhengeri, (6) Gicumbi/Byumba, (7) Gatuna; **Eastern Province:** (8) Nyagatare, (9) Kagitumba, (10) Rwamagana, (11) Ngoma/Kibungo, (12) Nyamata/Bugesera, (13) Ruhuha/Ngenda; **Southern Province:** (14) Muhanga/Gitarama, (15) Huye/Butare, (16) Nyamagabe/Gikongoro; and **Kigali City:** (17) The Capital).*

Moreover, other samples of antimalarial medicines were received from Kinshasa (university of Kinshasa, D.R. of Congo) and Cotonou (University of Abomey Calavi, Benin) in the frame of scientific collaboration including the University of Liege (Belgium) in the project of fighting against counterfeit medicines.

4. RESULTS AND DISCUSSIONS

4.1. Adaptation of the method for the analysis of quinine, resorcinol and major cinchona alkaloids in different pharmaceutical forms

A simple isocratic method for the analysis of quinine, resorcinol, and major cinchona alkaloids in different pharmaceutical forms was optimized thanks to the adaptation of other methods. In fact, from literature search, we identified two interesting references [18] [19] from which we did necessary adaptations with our regular mobile phase composed by methanol and ammonium formate buffer. Hence, we decided to run some experiments around the practical conditions of the two reference methods as summarized in **Table 1**; and we got

satisfactory results at 10 mM ammonium formate buffer pH 2.8 and methanol (60:40, v/v) as illustrated in **Figure 3** for the analysis of quinine, dihydroquinine and cinchonidine generally tested in quinine tablet forms [17]; and for the analysis of quinine, resorcinol, cinchonine, cinchonidine, quinidine, and dihydroquinine in injectable forms, the optimum proportions of mobile phase was found at 70:30, v/v for 10 mM ammonium formate buffer pH 2.8 and methanol, respectively. **Figure 4** illustrates the case of simultaneous separation of the six analytes in quinine resorcin solutions.

This method was also geometrically transferred to shorter analytical columns in order to reduce the analysis time and therefore increase the sample analysis throughput as well as reducing the consumption of the mobile phase.

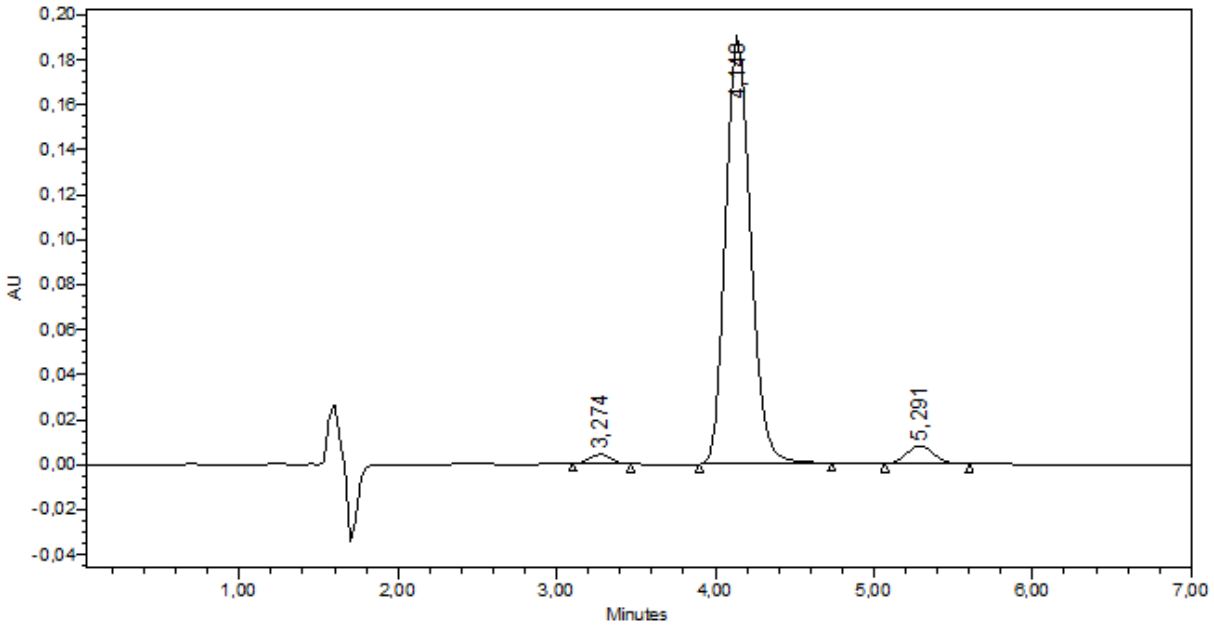


Figure 3 *Isocratic method for the analysis of quinine and major cinchona alkaloids in tablet pharmaceutical forms.* ***Experimental conditions:*** *Column: Zorbax C8, 150 × 4.6 mm, dp: 5 µm; Flow: 1 mL min^{-1}; T°: 35°C; λ = 230 nm; Isocratic elution with methanol and 10 mM ammonium formate buffer pH 2.8 (40:60, v/v).* ***Elution order:*** *Cinchonidine (≈ 3.274 min.), quinine (≈ 4.140 min.), dihydroquinine (≈ 5.291 min.).*

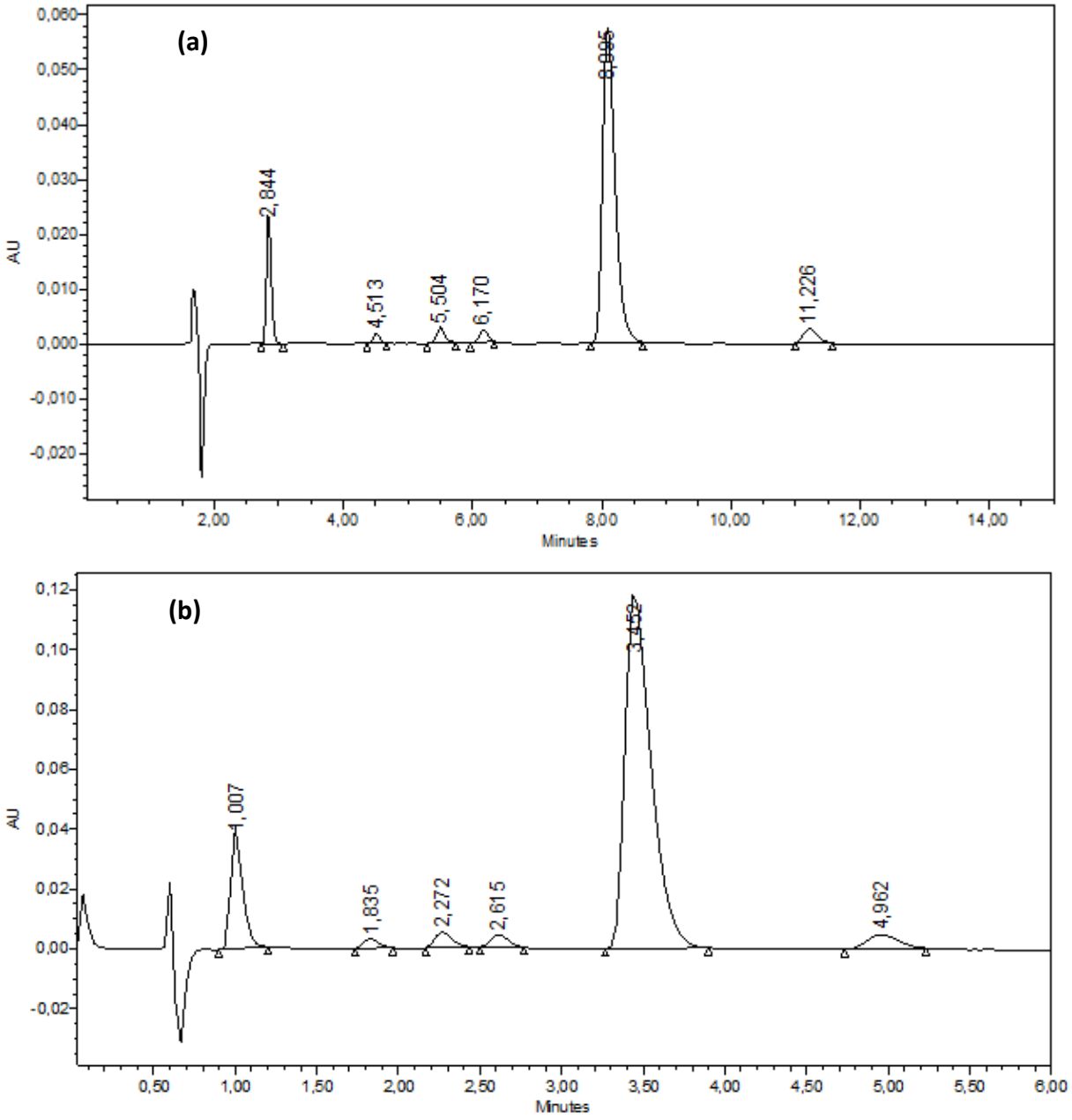


Figure 4 *Isocratic method for the analysis of quinine resorcin and major cinchona alkaloids in injectable solutions.* ***Experimental conditions:*** *Column: Zorbax C8, 150 × 4.6 mm, dp: 5 µm for chromatogram (a), and Zorbax C8, 50 × 4.6 mm, dp: 3.5 µm for chromatogram (b); Flow: 1 mL min^{-1}; T°: 35°C; λ = 230 nm; Isocratic elution with methanol and 10 mM ammonium formate buffer pH 2.8 (30:70, v/v).* ***Elution order*** *(identic to both chromatograms):* ***i) Chromatogram (a)****: Resorcinol (≈ 2.844 min.), cinchonine (≈ 4.513 min.), cinchonidine (≈ 5.504 min.), quinidine (≈ 6.170 min.), quinine (≈ 8.095 min.), dihydroquinine (≈ 11.226 min.);* ***ii) Chromatogram (b)****: Resorcinol (≈ 1.007 min.), cinchonine (≈ 1.835 min.), cinchonidine (≈ 2.272 min.), quinidine (≈ 2.615 min.), quinine (≈ 3.452 min.), dihydroquinine (≈ 4.962 min.).*

4.2 Adjustment of the method for the analysis of artemether/lumefantrine in tablet forms

Our earlier developed method in isocratic mode for the analysis of artemether and lumefantrine in tablet forms [16] was adjusted by improving the dissolution capacity of methanol on lumefantrine and therefore allowing to speed up the process of sample preparation by acidifying methanol at 0.2% w/v with phosphoric acid. Indeed, lumefantrine is practically insoluble in water, soluble in dichloromethane and chloroform, and slightly soluble in methanol but we privileged to dissolve it with the acidified methanol to avoid the use of chloroform or dichloromethane which are very chromophorous and capable of giving an additional peak in the chromatogram that would compete with lumefantrine and therefore reduce the detection of artemether which does not absorb well the UV light.

Moreover, the amount of lumefantrine is six times the amount of artemether in different medicines, and this is another challenge during sample preparation as there is need of having an effective solvent for both analytes that cannot compromise the chromatographic results. Hence, from the original method that had a run time of 16 minutes, we adapted the mobile phase proportions to 85:15, v/v of methanol and ammonium formate buffer pH 2.8 respectively, 0.7 mL min^{-1} of flow rate, and 25˚C of column oven using a Zorbax-Extend C18, 80Å, 100 × 4.6 mm (dp: 3.5 µm) analytical column. This allowed to reduce the analysis time from 16 to 6 minutes on a 100 × 4.6 mm column as illustrated in **Figure 5**, and to 10 minutes on a 150 × 4.6 mm column with the possibility to reduce the run time by changing the flow rate to 1.0 mL min^{-1} with the same mobile phase.

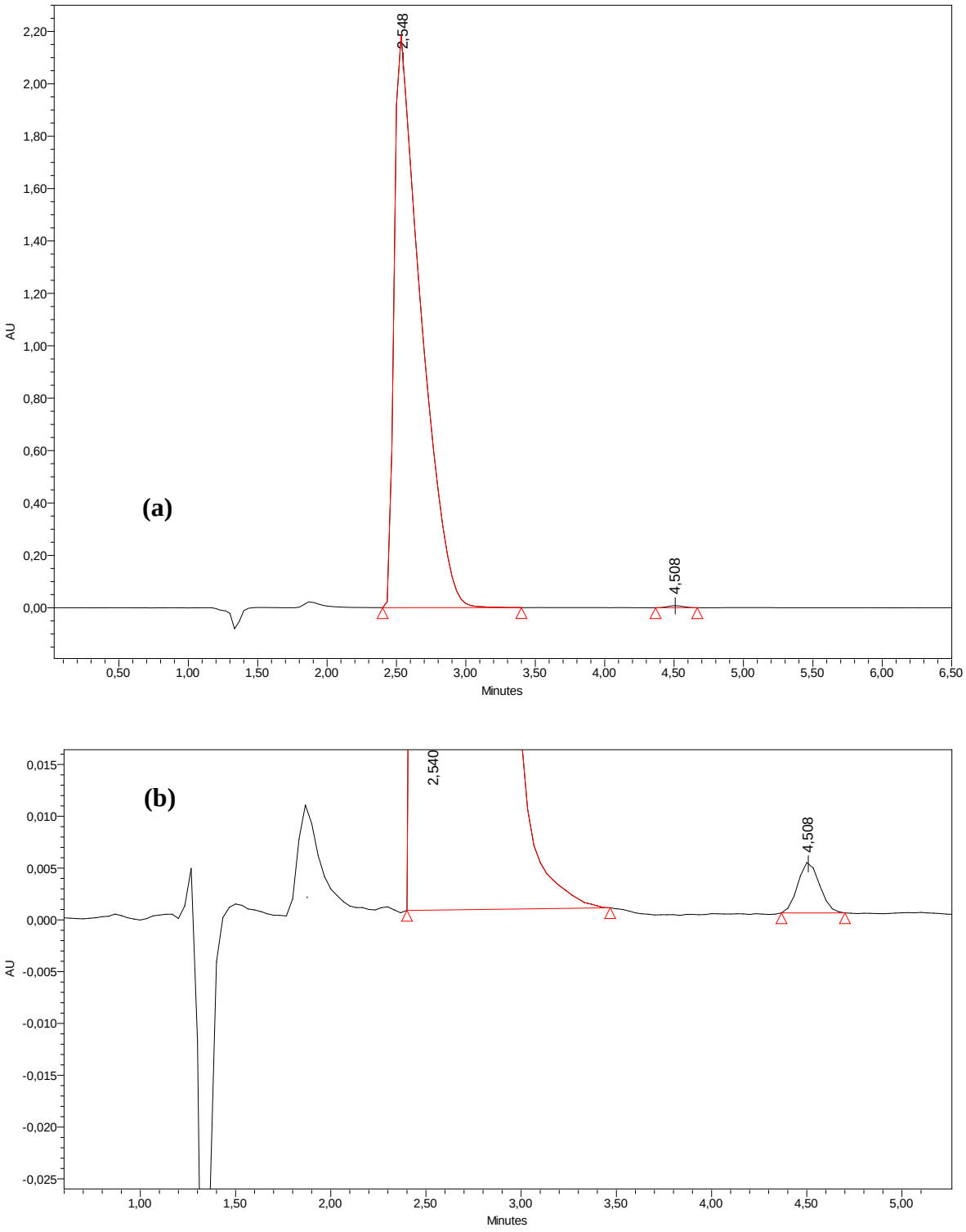


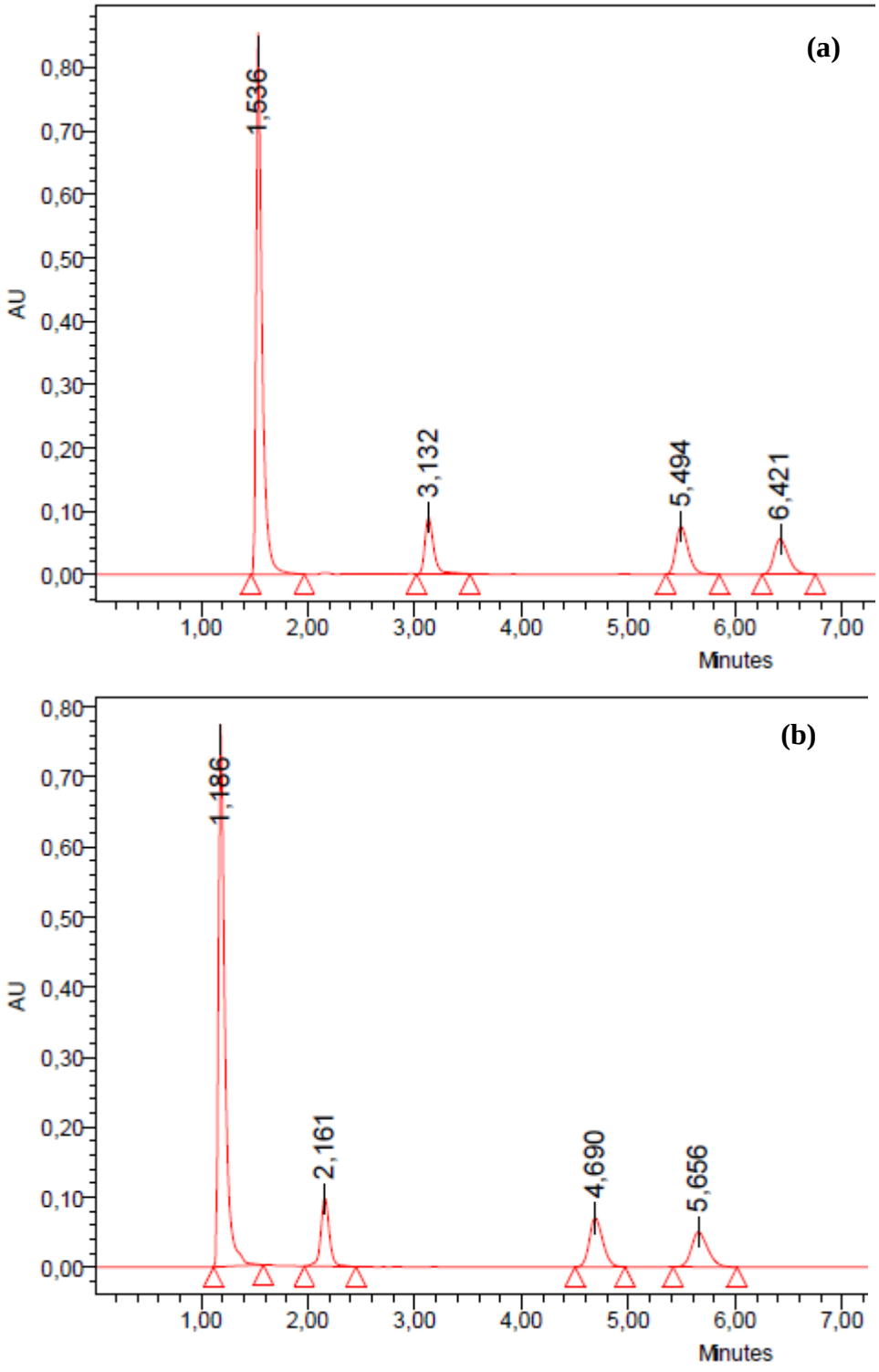
Figure 5 *Readjusted isocratic method for the analysis of artemether-lumefantrine in tablet forms.* ***Experimental conditions:*** *Column: Zorbax-Extend C18, 80Å, 100 × 4.6 mm, dp: 3.5 µm (entire and zoomed chromatograms to visualize artemether); Flow: 0.7 mL min^{-1}; T°: 25°C; λ = 210 nm; Isocratic elution with methanol and 10 mM ammonium formate buffer pH 2.8 (85:15, v/v).* ***Elution order:*** *Lumefantrine (≈ 2.548 min.) and artemether (≈ 4.508 min.) in Full chromatogram (a) and magnified chromatogram (b) for low abundant peak.*

4.3 Simple development and geometric transfer of an isocratic method for the analysis of arteether, artemether and artesunate in injectable forms

A simple isocratic method for the analysis of quinine, arteether, artemether and artesunate was developed thanks to a simple systematic approach based on sequential isocratic runs described in **Table 1** using octadecyl silane (ODS or C18) LC columns and the same mobile phase composed by methanol and ammonium formate buffer used in the previous methods development and optimization. In fact, we added quinine dihydrochloride to the three artemisinin compounds as another potential antimalarial medicine used in injectable forms to ensure that the method shall detect it in case of analyzing counterfeit medicines composed by one of the three artemisinin active ingredients.

Then, by following the planned systematic tests, we found better chromatographic results at higher proportions of the organic modifier, and we optimized the method at 90:10, v/v of methanol and 10 mM ammonium formate buffer pH 2.8; 0.7 mL min^{-1} to 1.0 mL min^{-1} of flow rate; 25˚C of the column oven compartment; and 210 nm of wavelength on a Zorbax Extend C18, 150 × 4.6 mm (dp: 5 μm) column; and we transferred the method to XBridge Shield RP18, 100 × 4.6 mm (dp: 3.5 μm), and Zorbax Extend C18, 50 × 4.6 mm (dp: 3.5 μm) columns to reduce the analysis time and mobile phase consumption, as well as allowing the increase of sample analysis throughput.

As illustrated in **Figure 6**, one can notice that the method has the ability of separating well the three artemisinin compounds, and that it can allow detecting any counterfeit of mixing them; but also the method was found capable of detecting quinine which is another potential injectable medicine from the three compounds.


(a)
AU
0,80
0,70
0,60
0,50
0,40
0,30
0,20
0,10
0,00
1,536
3,132
5,494
6,421
1,00
2,00
3,00
4,00
5,00
6,00
7,00
Minutes
(b)
AU
0,80
0,70
0,60
0,50
0,40
0,30
0,20
0,10
0,00
1,186
2,161
4,690
5,656
1,00
2,00
3,00
4,00
5,00
6,00
7,00
Minutes

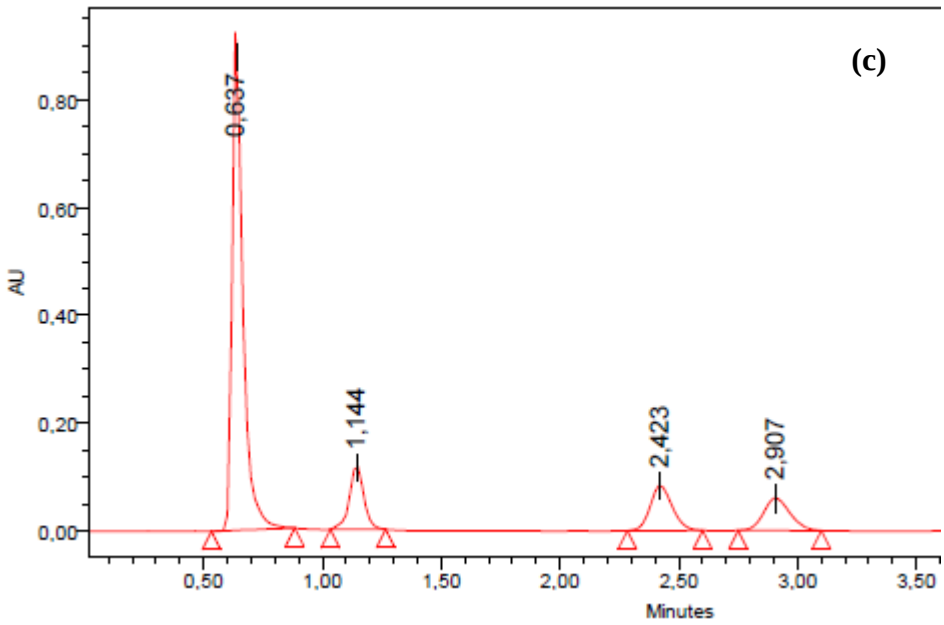


Figure 6 *Isocratic method for the analysis of quinine, arteether, artemether and artesunate in injectable forms.* ***Experimental conditions:*** *Column: (a) Zorbax Extend C18, 150 × 4.6 mm (dp: 5 µm); (b) XBridge Shield RP18, 100 × 4.6 mm (dp: 3.5 µm); (c) Zorbax Extend C18, 50 × 4.6 mm (dp: 3.5 µm); Flow: 1.0 mL min^{-1} for column (a), and 0.7 mL min^{-1} for column ((b) and (c)); T°: 25°C; λ = 210 nm; Isocratic elution with methanol and 10 mM ammonium formate buffer pH 2.8 (90:10, v/v).* ***Elution order:*** *(identic in in the three chromatograms).*
i) Chromatogram (a): Quinine (≈ 1.536 min.), artesunate (≈ 3.132 min.), artemether (≈ 5.494 min.), arteether (≈ 6.420 min.); ii) Chromatogram (b): Quinine (≈ 1.186 min.), artesunate (≈ 2.161 min.), artemether (≈ 4.690 min.), arteether (≈ 5.656 min.); iii) Chromatogram (c): Quinine (≈ 0.637 min.), artesunate (≈ 1.144 min.), artemether (≈ 2.423 min.), arteether (≈ 2.907 min.).

4.4. Methods validation

After methods optimization, it is necessary to demonstrate that the new methods are suit-for-purpose by providing accurate analytical results. In this regards, we have selected the method for analysis of artesunate powder for injection as a new antimalarial medicine in Rwanda aligned in the national list of essential medicines in 2015 [21] [22]. Moreover, this method can also help in detecting other arteminisin compounds such as arteether and artemether which are closely related to artesunate and which can be found in counterfeit or substandard artesunate medicines. Hence, the validation criteria as required by the International Conference on Harmonization (ICH) in its document Q2(R1) were considered namely: selectivity, trueness, precision (repeatability and intermediate precision), accuracy, linearity, limit of detection (LOD) / limit of quantitation (LOQ), and dosing range [7].

At first, we checked the selectivity of the method by checking whether there is no peak interference at the t_R of artesunate especially by other artemisinin compounds widely used in

pharmaceutical formulations i.e. arteether and artemether, and this criteria was satisfactory as illustrated in **Figure 7** since all peaks were well resolved.

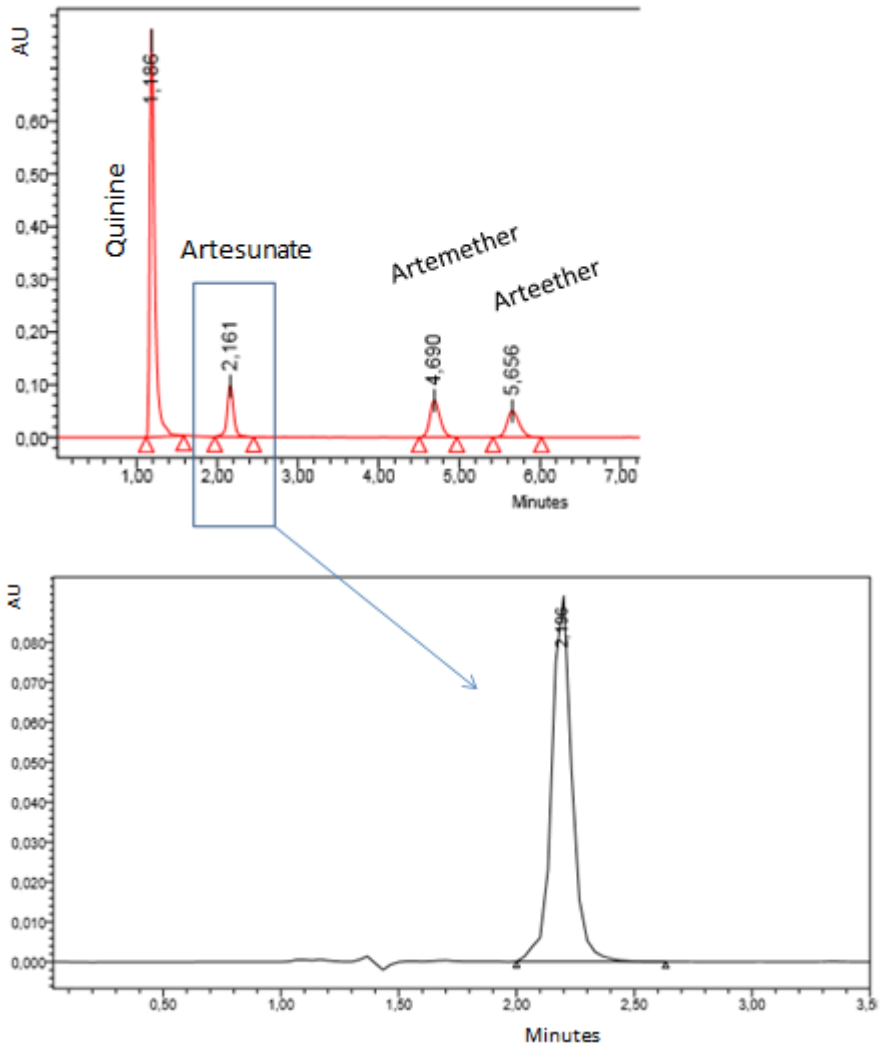


Figure 7 *Chromatograms of artesunate with other compounds (a) and artesunate alone in a sample solution (b).* ***Experimental conditions:*** *Column: XBridge Shield RP18, 100 × 4.6 mm (dp: 3.5 µm); Flow: 0.7 mL min^{-1}; T°: 25°C; λ = 210 nm; Isocratic elution with methanol and 10 mM ammonium formate buffer pH 2.8 (90:10, v/v).*

At second, we applied the concept of total error strategy represented by accuracy profiles as decision tool on the fit-for-purpose of the method for its intended use [8] [9] [10] [11]. By using the data of CS, the linear regression model was constructed and allowed obtaining the calculated result from VS. Then, an accuracy profile for artesunate was drawn as can be seen in **Figure 8** with the results of validation criteria summarized in Table 2. The acceptance limits were set at ±5.0% according to the European Medicine Agency (EMA) standard for finished pharmaceutical products [23], and the analytical results from this method comply automatically with the larger specifications of ±10.0% as per the International Pharmacopoeia

monograph for artesunate powder for injection [24]. Indeed, we choose the narrower acceptable limits to allow the method being suitable to both regional requirements (European and International Pharmacopoeias), and the end-user regulatory authority shall choose which limit to enforce.

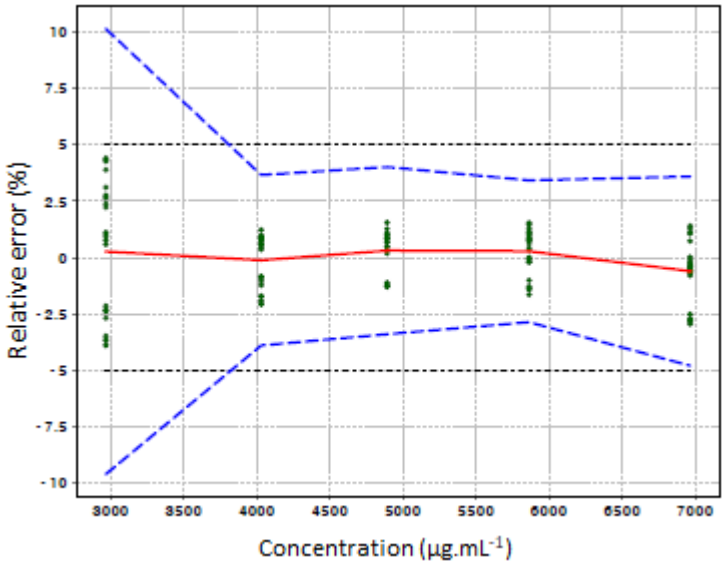


Figure 8 *Accuracy profile for quantitative method validation of artesunate powder for injection. The plain red line represents the relative bias, the blue dashed lines the 95% β-expectation tolerance limits and the black dotted lines the 5% acceptance limits. The green dots express the relative error of the back-calculated concentrations plotted with respect to their targeted concentrations. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).*

Then after, from the back-calculated results of VS that are the experimental ones, trueness of the method was assessed as it is the closeness of agreement between a conventionally accepted value (or reference value) that corresponds to the introduced concentrations of the analyte and a mean of experimental ones. We were able to get information on the systematic error that was found quite acceptable with relative biases between −0.1% and 0.3% compared to the two considered acceptable limits.

The method precision was also found acceptable since there was a closeness of agreement among measurements; here, the back-calculated results of VS obtained from multiple sampling of homogeneous samples of the analyte. The relative standard deviation values for repeatability and for intermediate precision at the target of 100% concentration level were acceptable with a maximum of 0.3%for repeatability, and 3.2% for intermediate precision.

To demonstrate the method linearity, we assessed the relationship between the back-calculated results of VS (experimental ones) against the introduced concentrations. The linear regression model was fitted on the two types of concentrations, with a good linearity of the results illustrated in **Table 2** by the slope close to 1.

Moreover, method accuracy taking into account the total error, i.e. systematic and random errors, was assessed from the accuracy profile shown in **Figure 8**. In addition, as shown in **Table 2**, the relative β-expectation tolerance intervals are within a range of [−4.8%, 4.0%] except level 1 which is between −9.6% and 10.1%. Hence, as the lower and upper tolerance bounds are included within the acceptance limits for the targeted concentration level of 5000 μg mL^{-1}, one can guarantee that at least 95% of future experimental results will fall within the acceptance limits [25].

We estimated also the limit of detection (LOD) that is the smallest quantity of the targeted substance that can be detected, but not accurately quantified in the sample. The computed value was 124.1 μg mL^{-1}.

The lower limit of quantitation (LLOQ) which is the smallest quantity of the targeted substance in the sample that can be assayed under experimental conditions with well-defined accuracy was calculated and its value is 3820 μg mL^{-1} vs. the upper limit of quantitation (ULOQ) which is the highest quantity of the targeted substance in the sample that can be assayed under the experimental conditions with well-defined accuracy was calculated at 6962 μg mL^{-1}. In fact, those limits of quantitation were obtained by calculating the smallest and highest concentrations beyond which the accuracy limits or β-expectation limits go outside the acceptance limits. Hence, the intervals between the lower and the upper limits where the procedure achieves adequate accuracy allowed us to set the “Dosing range”, again equivalent to [3820 μg mL^{-1} to 6962 μg mL^{-1}].

Finally, the uncertainty which is a parameter associated with the result of a measurement that characterizes the dispersion of the values that could reasonably be attributed to the measurand was calculated, and its relative expanded value (%) was found less than 3.1% on the four concentration levels except level 1.

Table 2.

Summary of the validation criteria for artesunate powder for injection

Validation criteria		Conc. (µg mL^{-1})	Artesunate
Trueness:		3000.0	7.9 *(0.3)*
Absolute bias (µg mL^{-1})		4000.0	-4.8 *(-0.1)*
(Relative bias (%))		5000.0	15.0 *(0.3)*
		6000.0	16.3 *(0.3)*
		7000.0	-42.0 *(-0.6)*
Precision:		3000.0	0.3 / 3.3
Repeatability (%) /		4000.0	0.2 / 1.3
Intermediate precision (%)		5000.0	0.2 / 1.1
		6000.0	0.2 / 1.1
		7000.0	0.2 / 1.4
Accuracy:		3000.0	2680 – 3264 *[-9.593, 10.13]*
95% β-expect. tol. int. (µg mL^{-1})		4000.0	3870 – 4174 *[-3.9 – 3.7]*
[Rel. 95% β-expect. tol. int (%)]		5000.0	4722 – 5084 *[-3.4 – 4.0]*
		6000.0	5692 – 6060 *[-2.9 – 3.4]*
		7000.0	6628 – 7211 *[-4.8 – 3.6]*
Linearity :	Slope		0.9916
	Intercept		39.49
	R^2		0.9975
Dosing range :	Lower LOQ (µg mL^{-1})		3820
	Upper LOQ (µg mL^{-1})		6962

4.5. Application of the methods

The validated method for analysis of artesunate powder for injection was applied in the analysis of 3 different batches found in public hospitals in Rwanda during our sampling, whose results are presented in Table 3. The other methods were also applied in the analysis of artemether/lumefantrine in different samples of medicines sampled in the D.R. Congo (9 samples), Rwanda (13 samples) and Benin (1 suspected counterfeit) as reported in another article submitted to the Current Drug Safety Journal - Bentham Science Publishers [26], and the method for quinine and major cinchona alkaloids was used in the analysis of suspected counterfeit quinine tablets as reported in the American Journal of Analytical Chemistry [27].

The methods developed in Belgium on a LC Waters 2695 were successfully tested to Rwanda on two other LC systems namely Agilent 1200 series and Agilent 1260 series both equipped with diode array detector (DAD) from Agilent Technologies (Boblingen, Germany) and Chemetrix (Agilent Technologies authorized distributor, Midrand, South Africa) respectively (results not shown).

Table 3. Assay results of the analyzed three batch samples of artesunate powder for injection coded A, B and C sampled in Rwanda. The results consist in the mean percentage of claimed nominal content and the relative standard deviation calculated on 3 independent sample solutions per batch. Specifications are set to 90.0% - 100.0% of the claimed nominal content (mg).

Drug sample	**A**	**B**	**C**
Claimed	60 mg	60 mg	60 mg
Assay in %	99.4 ± 0.5 %	99.4 ± 0.1 %	100.4 ± 0.4 %

5. Conclusions

Simple isocratic methods were developed thanks to methods adaptation or adjustments approach, and sequential systematic tests without passing through long and expensive DoE. The isocratic methods were privileged for their simplicity, short analysis time and high sample throughput, low mobile phase consumption, and adaptability to LC systems equipped with simple or gradient pumps. In this regard, we developed a generic method for the analysis of: (i) artesunate, arteether and artemether in injectable forms; (ii) artemether and lumefantrine in tablets; and (iii) quinine-resorcin with major cinchona alkaloids (cinchonine, cinchonidine, quinidine, and dihydroquinine).

The analytical method for artesunate powder for injection derived from the generic method for artesunate, arteether and artemether was fully validated thanks to the strategy of total error and accuracy profile approach in accordance with the criteria of ICH Q2 (R1) guidelines; and finally, the methods were applied in the analysis of real samples of artemether/lumefantrine medicines, artesunate powder for injection, and counterfeit quinine tablets.

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IV.4 SECTION 4 - Detection of poor quality artemisinin-based combination therapy (ACT) medicines marketed in Benin using simple and advanced analytical techniques

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IV.4.1 Preamble to Article 4

The fight against counterfeit medicines requires among others, a collaboration between several sectors working in the pharmaceutical field such as customs services, medicines agencies ... The scope of the collaboration can be international, but could be more effective at the regional level given the possible similarities of cases of counterfeiting and the proximity of interventions.

In this section, we present a case of collaboration among three countries in south, namely the Benin, the DRC and Rwanda, and Belgium in north, all with the aim of deploying analytical methods to monitor the quality of artemisinin-based combination therapy (ACT). As a reminder, these medicines are widely used for the treatment of malaria as first line (as mentioned by the WHO). Several trademarks of those ACTs, dosages forms and strengths are marketed, and need to be controlled for compliance with the quality specifications.

Various samples were obtained from the three aforementioned countries and analyzed according to a decision tree ranging from simple to complex analytical tests. Among the results were found out-of-specification cases, and an alarming case of fake Coartem that did not contain any of the two expected active ingredients.

IV.4.2 Article 4

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RESEARCH ARTICLE

Detection of Poor Quality Artemisinin-based Combination Therapy (ACT) Medicines Marketed in Benin Using Simple and Advanced Analytical Techniques

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ABSTRACT

Background: Poor quality antimalarial medicines still represent a threat to the public health, especially in Sub-Saharan Africa who bears a disproportionate share of the global burden of malaria. It is essential and urgent to strengthen mechanisms against counterfeit medicines. One of the approaches is regular market surveillance through quality controls.

Methods: 12 samples of artemether/lumefantrine were collected from formal and informal drug sellers in Cotonou (Benin) as well as additional other similar samples from Rwanda (13 samples) and from D.R. Congo (9 samples). Thin Layer Chromatography (TLC) as classical and simple identification test was applied in Benin while an analytical chemistry laboratory in Belgium (ULg, Pharmacy Department) was asked for further analyses with HPLC and Raman spectroscopy using a developed and validated HPLC method for rapid analysis of artemether/lumefantrine.

Results: The results obtained in Belgium confirmed the lack of the two active ingredients in the suspected sample of ACT medicine from Benin whereas some samples from Rwanda and D.R. Congo were found to present risk of substandard drugs either for under-dosing or over-dosing.

Conclusions: Counterfeit/falsified of artemisinin-based combination therapy (ACT) medicines are really scourge that needs to be fought through strong collaboration between public health authorities and appropriate quality control laboratories.

1. INTRODUCTION

Artemether/Lumefantrine (AL) was the first fixed-dose artemisinin-based combination therapy recommended and pre-qualified by the World Health Organization (WHO) for the treatment of uncomplicated malaria caused by *P. falciparum*. It is currently recommended as first-line treatment for uncomplicated malaria in several countries [1].

The efficacy of AL in Africa and South America remains high, with treatment failure rates generally below 10%. Currently, 40 countries in Africa and 6 countries in South America are using this combination as first or second-line treatment. In Angola, Burkina Faso, Gambia, Ghana, Malawi, Niger, Nigeria and Zimbabwe, isolated studies conducted between 2006 and 2013 have shown treatment failure rates with AL above 10% [1].

In 2014, global financing for malaria control increased from an estimated US$ 0.96 billion in 2005 to US$ 2.5 billion. As far as spending on malaria control commodities (artemisinin-based combination therapies “ACTs”, insecticide-treated bed nets “ITNs”, insecticides and spraying equipment for indoor residual spraying “IRS”, and rapid diagnostic tests “RDTs”) are concerned, it is estimated to have increased over the past 11 years, from US$ 0.04 billion in 2004 to US$ 1.6 billion in 2014, and accounted for 82% of international malaria spending in 2014. ACTs represented 25% of total commodity spending in that year [1].

The therapeutic benefits of ACTs are their high efficacy, rapid action and the reduced likelihood of resistance development. In order to make best use of this medicine combination, it is very important to address issues of quality. According to the WHO, 200,000 deaths over one million that occur from malaria annually would be avoidable if the available medicines were effective, of good quality and used correctly [2]. Unfortunately, recent studies report the circulation of higher levels of poor quality antimalarial medicines (i.e. counterfeit / falsified, substandard, and degraded) in most malaria endemic countries, and therefore highlighting the need of strengthening national drug regulatory authorities through quality assurance and

quality control (QC) systems, as well as regular market surveillance in order to secure the public health [3].

Analytical chemistry applying separative screening methods, especially with liquid chromatography (LC), are suitable in fighting against the spread of counterfeit/falsified and other poor quality medicines in this context [4; 5].

In the framework of fighting against poor quality antimalarial medicines marketed in Benin, especially ACTs; we conducted several analyses to assess the quality of suspected samples. At first, thin layer chromatography (TLC) was applied as simple identification technique allowing to detect the presence or not of the claimed active ingredients prior to their assay. Then, further analyses including HPLC and Raman spectroscopy were carried out to gain more information on suspected samples and similar samples from Rwanda and DRC in order to aware their status. The applied HPLC method was developed and validated in-house during our previous study for rapid analysis of AL [5].

2. MATERIALS AND METHOD

2.1. Samples

Drug sampling was done in Cotonou, the economic capital of Benin. This southern city concentrates most health facilities (about 45% of medical units installed in urban areas), the big markets in the country [6] including the international market Dantokpa. The samples, here tablets were collected from formal markets in pharmaceutical establishments (pharmacies opened to public) and informal drug sellers. Twelve samples were collected: 3 from pharmacies opened to public (formal system) and 9 from informal vendors (informal market system). They were submitted to visual inspection and instrumental analyses.

Additional samples were collected from Rwanda (13 samples) and D.R. Congo (9 samples), and analyzed at the University of Liège (ULg) / Pharmacy Department - Laboratory of analytical chemistry (Belgium).

2.2. Visual Inspection

According to the WHO guidelines for visual inspection of samples [7], we focused on the control of packaging, of labeling and of physical appearance of the pharmaceutical forms namely the specific size, the shape and the color in order to assess any abnormal presentation that can be an indice of potential counterfeiting or deterioration [8]. All samples were further submitted to instrumental analyses to check the presence or not of the active ingredients, the presence of undeclared compounds in the formulation, impurities or sub-degradation compounds when detectable as well. The **Figure 1** summarizes the analytical steps that were followed during this study. Additional pharmacopoeial tests were done namely uniformity of weight and disintegration tests [9, 10].

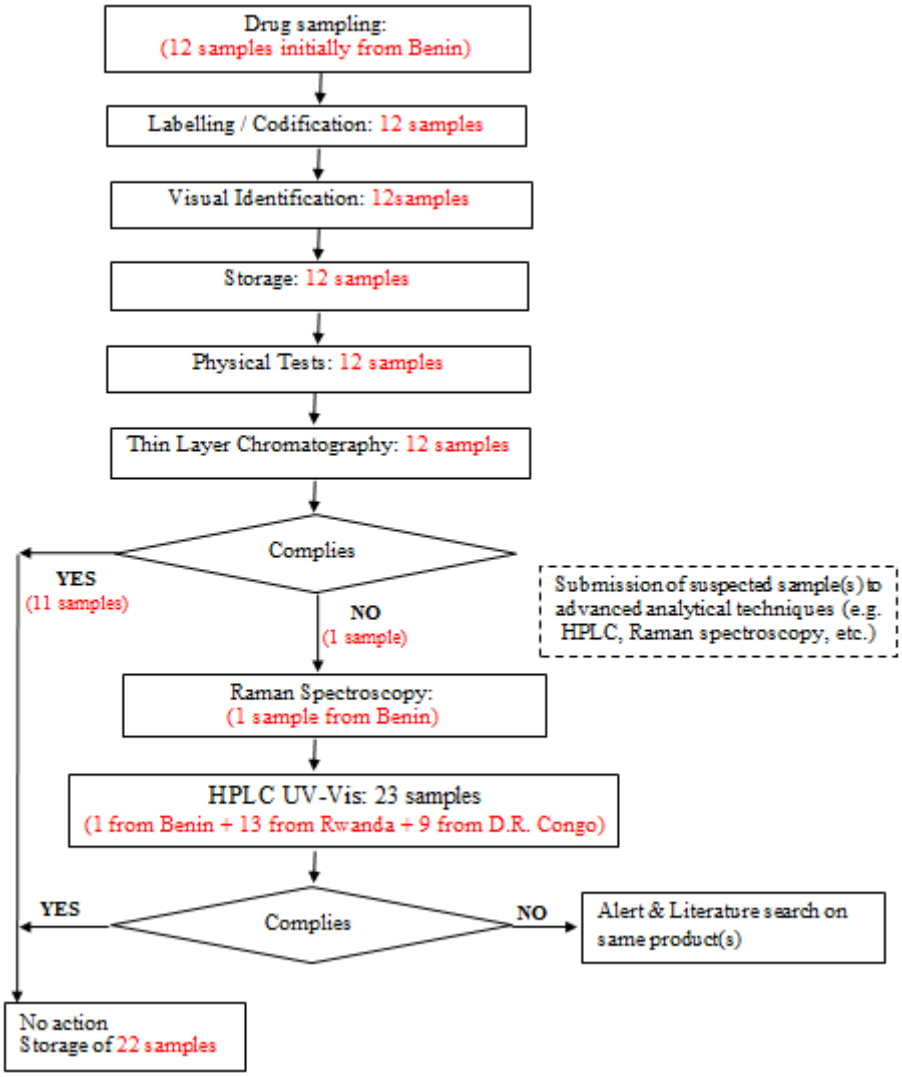

Figure 1: Flowchart of the analytical and decisional approach

2.3. Instrumental Analysis Methods

2.3.1. Chemical and Reagents

Methanol (HPLC gradient grade), anhydrous acetic acid, toluene, ethyl acetate, concentrated sulfuric acid (96%), formic acid (98% - 100%) and orthophosphoric acid European Pharmacopoeia grade (85%) were purchased from Merck (Darmstadt, Germany) and ammonium formate (99%) was from BDH Prolabo (Almere, Netherlands).

Ultrapure water was obtained from a Milli-Q Plus 185 water purification system from Millipore (Billerica, MA, USA). Antimalarial drugs containing AM (20, 40 or 80 mg) and LF (120, 240 or 480 mg) were purchased from formal and informal drug sellers.

2.3.2. Thin Layer Chromatography

We used an adapted standard operating procedures (SOP) provided with the MiniLab®. The GPHF-Minilab® is a mini-laboratory used in developing countries by medical stores and hospital managers, drug inspectors, and other authorities to detect counterfeit and substandard pharmaceuticals to provide basic quality controls. A model is available at the Benin National Quality Control Laboratory of Medicines and other Health Commodities, Ministry of Health (Cotonou). We have adapted our protocol from this model. Each sample was monitored on TLC plates as stationary phase made of silica gel 60F254S (Merck; Germany) while the mobile phase was a mixture of (toluene/ethyl acetate/anhydrous acetic acid (9: 2: 1; v/v/v). The lamp detection-UV was done at 254 nm for lumefantrine while for artemether, we used a spray reagent (methanol/sulfuric acid concentrated (19:1; v/v) followed by 5 minutes heating.
A stock solution of reference standards of artemether (AM) (2 mg/mL) and lumefantrine (LF) (12 mg/mL) was prepared in methanol acidified by anhydrous acetic acid (9:1; v/v). Dilutions were performed in methanol in order to obtain solutions at 3 different concentration levels:

Level 1 (60%): 1.2 mg/mL (for AM) – 7.2 mg/mL (for LF);
Level 2 (80%): 1.6 mg/mL (for AM) – 9.6 mg/mL (for LF);
Level 3 (100%): 2 mg/mL (for AM) - 12 mg/mL (for LF);

For the samples, powdered (n = 10 tablets) or liquid (n = 2 bottles) portions were taken and treated in the same way as reference solutions to give final expected concentrations of 2 mg/mL (for AM) and 12 mg/mL (for LF). The solutions were freshly prepared and protected from light. They were centrifuged at 3500 rpm for 5 minutes at 25°C. The supernatant was used for TLC analysis. All samples were analyzed in duplicate on the TLC plate.

For identification purposes, we compared the Rf value and the spot intensity of the standard reference against those observed with the sample solution.

2.3.4. Raman Spectroscopy Method

Intact tablet samples vs. intact small quantities of reference materials were used. The samples were analyzed with LabRAM HR Evolution (Horiba scientific, Kyoto, Japan) instrument equipped with two-dimensional Newton 970 front-illuminated EMCCD detector (1600 × 200 pixel sensor) (Andor Technology Ltd, Belfast, UK), Leica 50× Fluotar LWD objective and 785 nm laser with a power of 45 mW (XTRA II single frequency diode laser, Toptica Photonics AG, Munich, Germany). A 300 gr/mm grating was used to record the spectra in the spectral range of 1853 - 464 cm^{-1}.

The confocal slit-hole was fixed at 200 μm; each spectrum results from two acquisitions per 1 second. The spectra were collected with the LabSpec 6 (Horiba Scientific) software. Once acquired, the spectra were baseline corrected using the Asymmetric Least Squares (AsLS) algorithm with a λ value of 10^5 and a p-value of 10^{-3}. The baseline corrected spectra were the analyzed by MCR-ALS with non-negativity constraints on both concentration and spectra. Two spectra were resolved [11, 12]. The baseline corrected spectra were then scaled between 1 and 0 and compared to the spectral database. All spectrum processing and correlation coefficient computations were performed using Matlab R2013a software (The Matworks, Natick, MA, USA) and in-house routine coding.

2.3.3. HPLC analyses

HPLC analyses were performed according to the methods described in [5] with some few adaptations. They were carried out on a Waters 2695 Alliance (Waters, Milford, MA, USA) separation module coupled to Waters 2996 photodiode array (PDA) detector (Waters). The

system was controlled with Empower 2.0 software (Waters). The chromatographic separation was done in a Zorbax SB-C18 (dp 3.5 µm) column (100 mm × 4.6 mm ID) maintained in a controlled compartment at 25˚C, applying as mobile phase an isocratic mixture of methanol and 10 mM ammonium formate buffer adjusted to pH 2.8 with formic acid (85:15, v/v); the pump flow rate was 0.7 mL/min. The sample solutions were maintained in a controlled compartment at 15˚C. The injection volume was 6 µL. The UV detection wavelength was fixed at 210 nm. However, the UV spectra were recorded online from 210 nm to 400 nm.

3. RESULTS AND DISCUSSIONS

Our sampling (n = 9) took place at the biggest unofficial market "Adjegounlé" (located at the international market Dantokpa) with two additional markets in Cotonou: Fifadji and Gbegamey. Three (n = 3) samples were collected from official pharmacies.

Visually, none of the samples was expired at the day of purchase; only three inserts were written in French (official language in Benin) while all others were written in English. Irregularities were observed in the physical appearance of several samples such as small brown or black spots leading to suspect contaminants. One batch presented a non-homogeneous color. Moreover, among the samples from Benin, we noticed a sample not containing the indicated quantity of blisters probably due to the bad habit of patients who buy incomplete doses for their medication and that the missing blister was already sold earlier. All samples (n = 10) met the requirements for the uniformity of weight test (when weighed singly, the deviation of the individual masses from the average mass has not exceed 5%) [10]. One sample failed the disintegration test fixed at 30 minutes for film coated tablets [9], and the tablets were not yet disintegrated after 3 hours.

The twelve samples were coded for confidentiality, and were submitted to analysis with TLC. Eleven out of twelve samples were found to contain AL. Indeed the Rf value and the spot intensity of these two active ingredients were equivalent and similar to those of the standard solutions. The remaining sample (**Figure 2**) failed to that test (i.e. Coartem®; Batch number F2261; Manufactured date: 01/2014; Expired date: 02/2018; collected from the market of Fifadji). It corresponds to the sample that failed the disintegration test (time over 3 hours), thus it was suspected to contain no active ingredient after two tests failures.

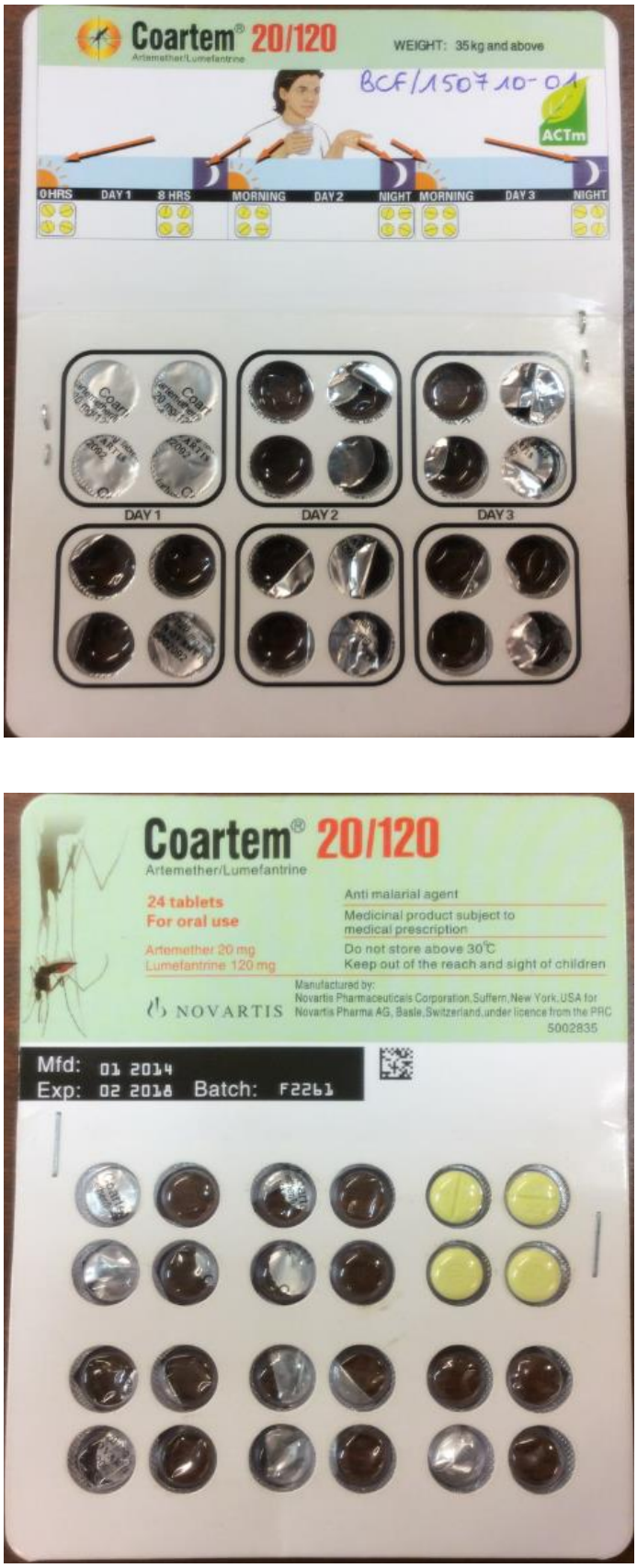

Figure 2: Suspected Coartem® 20/120 sample (BCF/1507 10-01) having failed TLC and disintegration tests

In Benin, a pilot study conducted in 2010 on the quality control of some antimalarial drugs sold in the same unofficial market Adjégounlè (Dantokpa market), reported that 2/3 of artemether- lumefantrine samples failed with the standards of the pharmacopoeia [13].

Thus we decided to focus on the sample having failed preliminary tests in our study, and submitted it to further analyses with advanced analytical techniques such as Raman spectroscopy and HPLC available at our partner Laboratory of Analytical Chemistry (GMP certified and WHO prequalified) based at the University of Liege (Belgium).

Raman spectroscopic analysis was done to check whether the suspected Coartem® contains a known compound, by comparing its spectral characteristic to a spectral database created for this purpose to generate candidate products that are likely to be in the composition of the suspected medicine. By comparing the sample to earlier AL recorded database, matching result are: artemether (r = 0.3343), lumefantrine (r = -0.0680) (see **Figure 3**). The computer system generated a list of compounds with related levels of likeliness to be in the composition of the sample (see **Figure 4**). The two best matches are: calcium carbonate (r =0.9458), potato starch (r = 0.9184), which are normally known as inactive pharmaceutical excipients. Some components from Raman spectroscopy best matches suspected to be in the sample are usually used in that formulation. However, we were not able to identify the coloring agent to yellow imitating the color of lumefantrine.

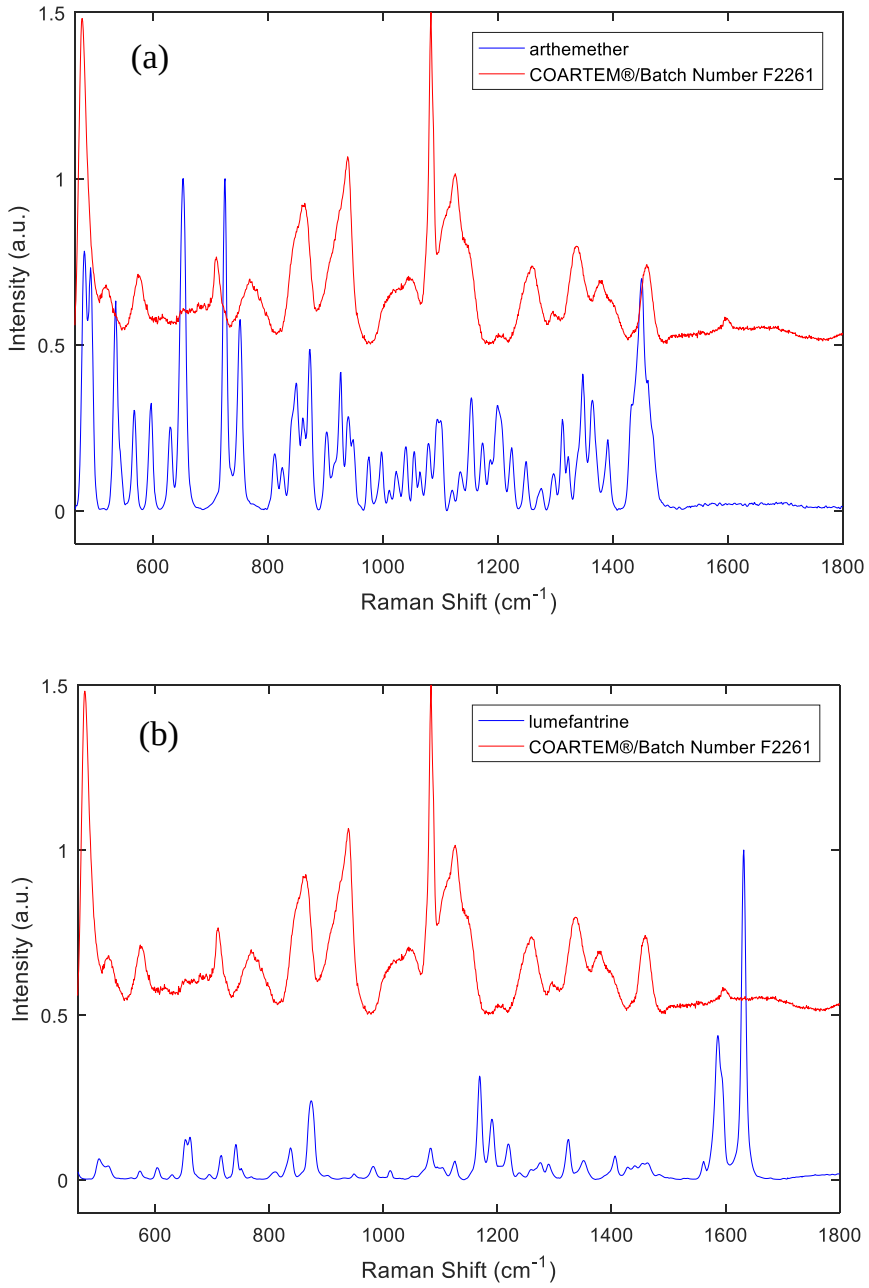

Figure 3: Raman Spectra: (a)- artemether spectrum in blue vs. sample spectrum in red; (b) - Lumefantrin spectrum in blue vs. sample spectrum in red. The spectra were offset for clarity

On the other hand, as illustrated in **Figure 5,** HPLC results confirmed that none of the two active ingredients was detected in the sample. Hence, by considering all previous analyses, we concluded that the Coartem® 20/120, batch number F2261, Mfd: 01 2014, Exp: 02 2018 is a counterfeit containing no active ingredient.

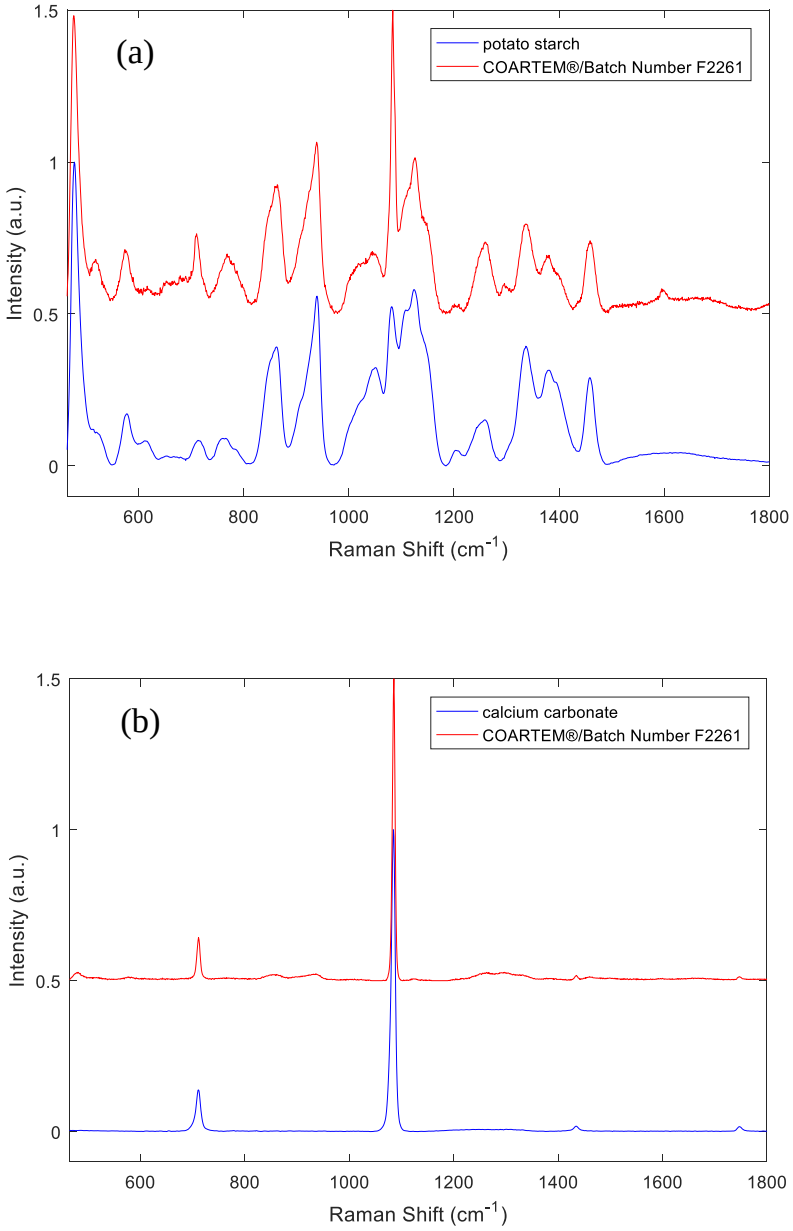

Figure 4: Spectroscopy Raman results. Compounds with related levels of likeliness to the sample: (a) for potato starch (r = 0.9184) and (b) calcium carbonate (r= 0.9458). The spectra were offset for clarity.

Furthermore, we conducted a literature search on the same batch product to see whether there is an alert issued about the same product, or a reported study. We were very interested with an article published in Malaria Journal on "Assessing the quality of anti-malarial drugs from Gabonese pharmacies using the MiniLab®" [12]. This field study reported 2 samples that failed the test, and one of the two samples is the same fake Coartem we sampled in Cotonou (Benin). However, even if the two products have a common batch number (i.e. F2261), brand name (Coartem®), and manufacturer (Novartis), they have totally different manufacture and

expiry dates i.e. Mfd: 01 2012, Exp: 02 2016 (found in Gabon) vs. Mfd: 01 2014, Exp: 02 2018 (found in Benin).High performance liquid chromatography was used to confirm the absence of APIs in the AL sample [14].

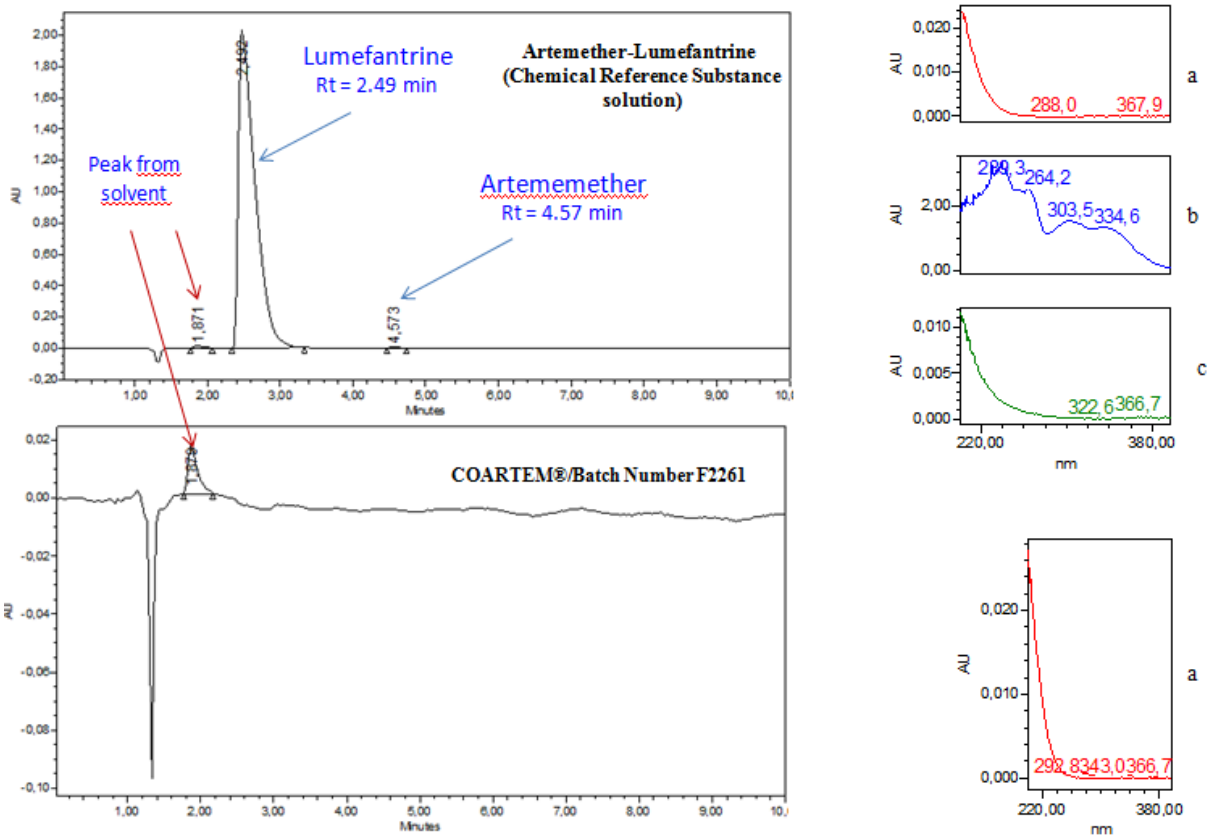

Figure 5: Chromatograms and UV-Vis spectra of sample BCF/1507 10-01 (Coartem®; Batch number F2261; Mfd: 01.2014; Exp: 02.2018; collected from the market of Fifadji; Benin); (a)- methanol spectrum; (b)- lumefantrin spectrum; (c)-artemether spectrum

In early 2013 a batch of falsified Coartem® was discovered in Yaoundé (Cameroon), containing no active pharmaceutical ingredients. The product failed the screening by the minilab® and was forwarded by an NGO to a WHO pre-qualified laboratory in Nairobi (Kenya) for more detailed analysis. Subsequent testing revealed that the product contained none of the claimed active pharmaceutical ingredients. The details of the falsified batches of Coartem® were: batch number: F1901, Exp: 01.2014 / Mfd: 01.2012; batch number: F2261, Mfd: 01.2012/Exp:01.2014. Surprisingly, this second one has the same batch number as the counterfeit samples found in Gabon and Benin, but the manufacture and expiry dates are totally different in the three cases. These are both genuine Novartis batch numbers which have passed their expiry dates. No genuine Coartem® bearing these batch numbers should

now be in circulation [15]. The same batch number is also in circulation in Mali [unpublished data]. The weakness of drug regulatory authorities in controlling the pharmaceutical supply chain is partly responsible of the prevalence of illicit medicines in resource limited countries. More efforts are still needed especially in those countries in order to effectively fight against the spread of poor quality medicines (counterfeit/falsified, sub-standards, and degraded), and therefore protect the public health against the terrible dangers they can cause. Sensitization, regular seize and destroy operations, prosecution of counterfeiters, regular market surveillance through quality controls, etc. are some urgent recommended actions; but above all, there is a real need of strengthening national drug regulatory authorities and set up a strong collaborations at national and international levels to overcome the porosity of borders in resource limited countries that can explain the easy circulation of fake medicines in those countries. In this context, further contacts were established with other research team members who are facing the poor quality medicines situation: Rwanda (East Africa); D.R. Congo (Central Africa). Similar samples were collected, 13 from Rwanda and 9 from D.R. Congo. Those samples were analyzed by the same HPLC method [5]. As can be seen in **Table 1,** all samples from these countries were compliant with the assay test specifications of 90.0% to 110.0% according to the United States and International Pharmacopoeias for both Artemether and Lumefantrine. However, 3 samples from D.R. Congo seemed to present a risk of under dose due to result (90.7%) closer to 90% limit and over dose due to results (109.4% and 108.4%) closer to 110% limits while the standard deviations were somewhat high. When we look on the survey study conducted by the WHO in six African countries [16] in which it was compared the outcomes of quality control laboratory testing and the GPHF-Minilab® screening, it was mentioned that GPHF-Minilab® can reliably detect grossly sub-standard samples, but should not be used as an independent testing resource or provide quantitative data except in conjunction with more sensitive techniques such as HPLC, etc. [17]. In the present study case, we supplemented our adapted TLC method (simple analytical technique, robust) by RAMAN spectroscopy and HPLC-UV Vis (advanced analytical techniques) to confirm the primary result. In this way, the results of our study support previous reports and publications on the issue of counterfeit medicines and drastic consequences they can cause to the public health. In fact, the problem is still critical, especially in resource limited countries, and there is real need to strengthen National Drug Regulatory Authorities of those countries in order to ensure that the distributed medicines are of the required quality, safety and efficacy.

Table 1: Results of Artemether (AM) and Lumefantrine (LF) assays in tablets samples from D.R. Congo and Rwanda

Samples from D.R. Congo (mean in %. ± SD; n=3)				Specifications (USP and Ph. Int) 90.0 – 110 %	Conclusion or remark for AM and LF
N°	**Samples codes**	**Content of LF (%)**	**Content of AM (%)**		
1.	RC001	98.2 ± 1.8	100.2 ± 0.2	OK	Compliant
2.	RC002	104.2 ± 1.5	97.7 ± 0.6	OK	Compliant
3.	RC003	**109.4 ± 1.8**	100.9 ± 1.3	OK	Risk of overdose for LF
4.	RC004	104.1 ± 0.8	96.6 ± 1.2	OK	Compliant
5.	RC005	107.8 ± 0.4	96.9 ± 0.6	OK	Compliant
6.	RC006	**108.4 ± 1.8**	100.1 ± 0.8	OK	Risk of overdose for LF
7.	RC007	94.6 ± 0.2	94.6 ± 0.5	OK	Compliant
8.	RC008	101.9 ± 1.2	**90.7 ± 1.8**	OK	Risk of underdose for AM
9.	RC009	106.7 ± 0.5	95.5 ± 0.8	OK	Compliant
Rwanda samples					
1.	K0904	94.1 ± 0.2	91.9 ± 0.7	OK	Compliant
2.	Q40093	97.1 ± 1.8	96.7 ± 0.1	OK	Compliant
3.	Q40037/ 7221596	92.7 ± 0.1	91.3 ± 1.0	OK	Compliant
4.	FA181	96.6 ± 0.8	93.5 ± 0.1	OK	Compliant
5.	Q40212	96.5 ± 1.2	98.8 ± 0.7	OK	Compliant
6.	Q40125	97.0 ± 0.3	97.7 ± 1.7	OK	Compliant
7.	X1684	97.7 ± 0.5	97.2 ± 0.5	OK	Compliant
8.	F3266	95.4 ± 1.1	94.0 ± 0.4	OK	Compliant
9.	DY11334909	95.9 ± 0.1	101.0 ± 1.4	OK	Compliant
10.	Q40353	94.1 ± 0.1	106.1 ± 1.9	OK	Compliant
11.	F3262	95.7 ± 0.6	95.8 ± 1.0	OK	Compliant
12.	Q30167	97.0 ± 0.1	97.7 ± 0.8	OK	Compliant
13.	P04013D	98.7 ± 0.8	107.7 ± 1.2	OK	Compliant

4. CONCLUSION

A counterfeit artemether/lumefantrine medicine was detected thanks to simple TLC testing adapted from GPHF-Minilab®, and disintegration test. The suspected fake medicine was submitted to Raman spectroscopy and HPLC analyses confirming the absence of both active ingredients in the sample collected at Fifadji market (Cotonou, Benin). The same fake batch of Coartem® was surprisingly found in Gabon, Mali and Cameroun but under different

manufacture and expiry dates in the three cases. However, even if the counterfeit ACT was found in Benin, Gabon, Mali, and Cameroon, and not found in the analyzed few samples from D.R Congo and Rwanda (Central Africa), it does not mean that the fake medicine would not be infiltrated to other countries including the Central Africa, and elsewhere where ACTs are widely used. Actually, recent reports on the quality of essential medicines keep to highlight the need of strengthening national and international strategic measures against the spread of poor quality medicines (counterfeit/falsified, substandard, and degraded), especially in resource limited countries where national medicine regulatory authorities (NMRA) are weak, and not able to fulfill their mission fully and efficiently.

CONFLICT OF INTEREST

The authors declare that they have no competing interests regarding the publication of this paper. There is no conflict with the manufacturer of the product being tested. FUNDING: Not applicable

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IV.5 SECTION 5 - Analytical Tools and Strategic Approach to detect poor quality medicines, identify unknown components, and timely alerts for appropriate measures: Case study of antimalarial medicines

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IV.5.1 Preamble to Article 5

As previously mentioned in section 4, international as well as regional collaboration is very important to fight counterfeit medicines. It is even more promising at the national level and at the local. In this context, this section has been developed with the intention of emphasizing the implementation of a strategic approach combined with analytical tools in the frame of detecting poor quality medicines, starting from the base (local level) up to top (international level).

Local collaboration helps in reporting suspected cases of non-compliance or falsification upon the complaints from consumers or medical staff, and international collaboration plays a very important role in analyzing the suspected cases with advanced analytical techniques that are more oriented to that purpose.

Thus, in this section, two cases of suspected counterfeit quinine tablets were analyzed, and advanced analytical techniques helped to identify the harmful nature of counterfeiting in the suspected medicines.

IV.5.2 Article 5

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Analytical Tools and Strategic Approach to Detect Poor Quality Medicines, Identify Unknown Components, and Timely Alerts for Appropriate Measures: Case Study of Antimalarial Medicines

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ABSTRACT

Nowadays, the circulation of poor quality medicines is becoming an alarming worldwide phenomenon with serious public health and socio-economic concerns. The situation is particularly critical in developing countries where drug quality assurance and regulatory systems for drug manufacturing, importation, distribution and sales are weak. A sustained vigilance on poor quality medicines that regroup counterfeit/falsified, substandard and degraded medicines is therefore required to ensure patient safety and genuine medicines integrity. A case situation is illustrated including a strategic approach and analytical tools that were found useful to detect poor quality medicines, identify unknown components, and timely alerts for appropriate measures against the spread of those harmful products. Several

suspected medicines randomly sampled in several strategic Rwandan areas were firstly check-controlled by means of visual inspection and then applying several analytical techniques from simple to more complex ones. The following medicines were studied: quinine sulfate tablets, artemisinin-based combination tablets, and artesunate powders for injection. Taking into account the pharmaceutical forms and the chemical characteristics, the following tests were applied: uniformity of mass, friability, disintegration, fluorescence, identification and assay. They were followed by more complex analytical techniques that allowed more comprehension of abnormal findings among which the presence of a wrong active pharmaceutical ingredient in quinine sulfate tablets which is mainly discussed in this paper to illustrate a strategic approach and various analytical tools that can be used in detecting and identifying unknown component in poor quality medicines.

1. INTRODUCTION

Over the past several years, the health of human beings is threatened not only by the old and new diseases, but also by the consumption of medicines that are not well known and mostly of poor quality. Today, more people than ever put their health in danger by purchasing and consuming medicines of doubtful quality. For example, up to 10% of all medicines sold worldwide are estimated to be counterfeit and in some parts of Africa and Asia the prevalence exceeds 30% as mapped in 2008 by Sanofi-Aventis [1]. The extent of damages caused by such drugs requires high collaborative endeavors at national and international levels to securize the public health, but also the trade of genuine products which are targeted by counterfeiters and deliberately falsified. Counterfeiters have gained access to sophisticated technologies that enable them to very closely imitate the packaging and labeling of legitimate generic or brand products [2]-[4]. However, in certain cases these products may contain unexpected products such as toxic ingredients, be ineffective or cause adverse effects due to incorrect concentration of the active ingredients or to the presence of wrong ones. In some circumstances, a drug that was initially of good quality may lose it for example by decrease of its potency or becoming toxic due to inappropriate storage conditions that lead to the appearance of impurities or degraded products. Newton *et al*. [5] distinguished three categories of poor quality medicines: 1) Counterfeit/falsified medicines which are illicit products maliciously produced and distributed; 2) Substandard also called out-of-specification "OOS" products which are genuine products generally produced in poor manufacturing conditions; and 3) Degraded medicines which are products improperly stored, and spoiled.

Detecting counterfeit or falsified medicines is a big challenge for healthcare professionals in charge of drug quality control. Strategies and specifications have been set up by several international organizations such as FDA, WHO, and EMA, defining requirements and procedures to follow for drug quality assessment. The first step to detect poor quality is usually the "*visual inspection*" on the physical characteristics of packaging, outer containers' labeling, closures, sealing, as well as the appearance of a medicine itself by its color, smell, consistency, etc. [2] [6]. Inspection of the storage conditions at the sampling sites such as the temperature, humidity, and exposure to light, is also one of the strategies recommended. During visual inspection, critical alterations are easily detected. Spoiled tablets, oral suspensions that harden, fluids leaking or containing particles/molds are quite enough signs to decide the non-conformity of a product without undergoing further laboratory tests. Nevertheless, this inspection needs a minimum and informative training to medicines end users for them to detect poor quality suspicions or visual evidences. Furthermore, laboratory analysis is of paramount importance during drug quality control for accurate identification and/or quantification of the active ingredients, and whenever possible of the excipients or impurities. That may necessitate using simple analytical techniques such as colorimetric tests, thin layer chromatography (TLC), UV-Vis spectroscopy or more complex techniques such as gas chromatography (GC), high performance liquid chromatography (HPLC), vibrational spectroscopy (Infrared, Near-infrared, Raman), mass spectroscopy (MS), nuclear magnetic resonance (NMR) spectroscopy, etc. [7]- [18].

The Republic of Rwanda is not spared from trafficking of poor quality medicines. Its government, highly aware of this situation, is continuously taking appropriate actions whenever feasible. For example, the regulatory system and analytical control laboratories are being implemented as well as the establishment of an Anti-Counterfeit Products and Illicit Pharmaceuticals Unit in the Rwanda National Police under the Department of Interpol [19] [20]. As a contribution to this endeavor, the aim of this study was to describe a strategic approach to detect poor quality medicines, identify unknown components, and as much as possible to timely communicate alerts to the competent organizations such as the National Medicine Regulatory Authorities (NMRAs), Ministry of Health (MoH), Healthcare Non-Governmental Organizations (NGOs), Manufacturers, Distributors, Health centres, and the WHO. For that purpose, several analytical techniques including simple and complex methods were involved targeting antimalarial medicines.

Figure 1 depicts a step-by-step guide to accomplishing drug quality assessment from sampling to instrumental analysis. The main steps are sampling, visual inspection, laboratory preparedness, sample preparation, reporting the results, and timely alerts for appropriate measures against the use of detected poor quality products. After alerting, there is a need to conduct advanced analysis with powerful analytical techniques in order to identify the nature and extent of the defective situation in the studied products especially in case of counterfeiting.

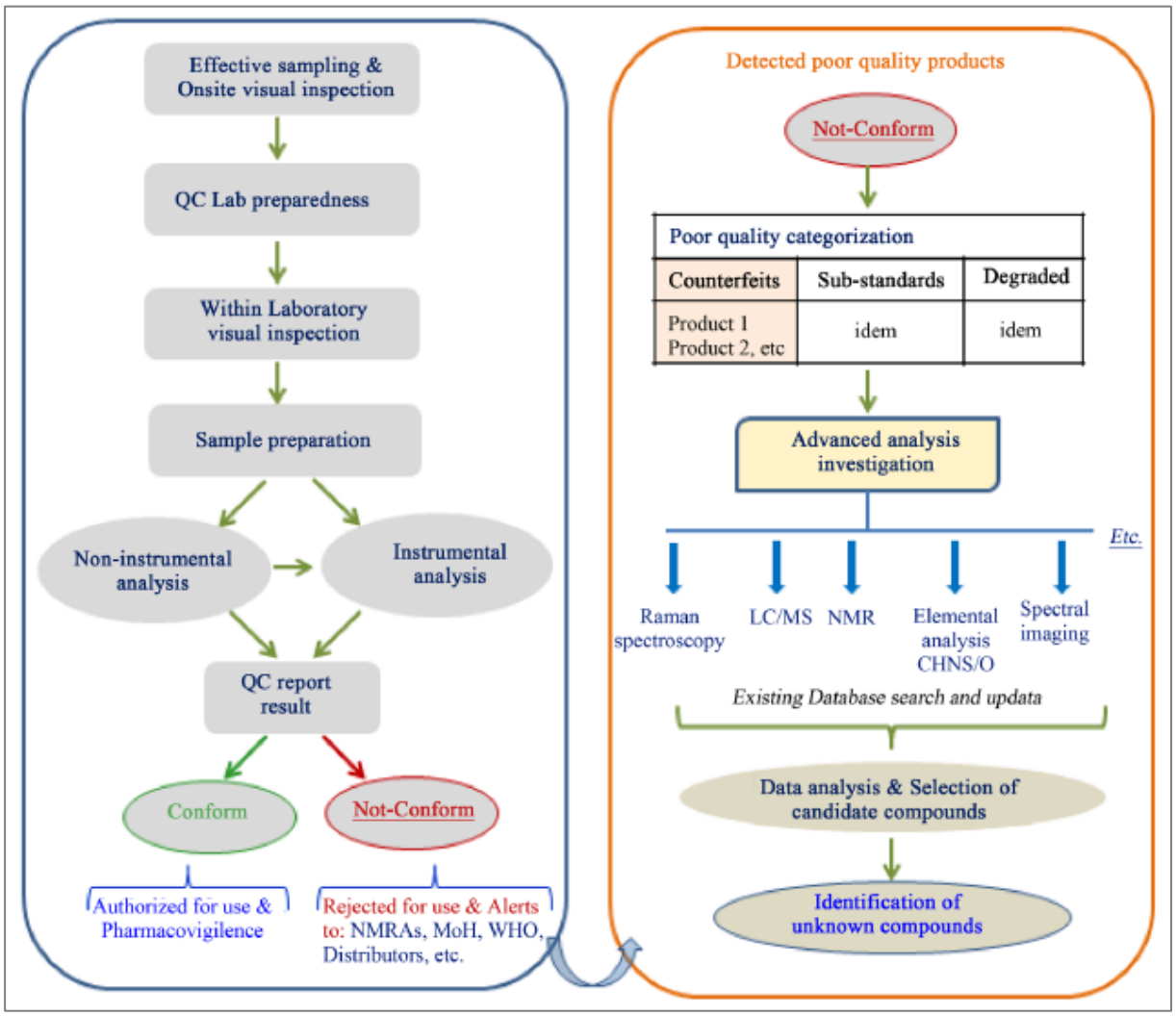


Figure 1. Step-by-step guide to drug quality control processing and alerting

2. Materials and Methods

2.1. Drug Sampling

As sampling sites, we privileged the countryside areas having intensive commercial activities due to border exchanges with Rwanda and neighboring countries namely, Burundi, Tanzania, Uganda, and D.R. Congo, and from which deceitful medicines could be infiltrated. For this study, we selected quinine and artemisinin-based preparations as most widely used antimalarial medicines in Rwanda. Different samples of quinine tablets 300 mg, artemether-lumefantrine tablets 20 mg/120mg, and artesunate powder for injection 60 mg were randomly purchased in retail pharmacies or received free of charges from public institutions operating in the selected areas. They were submitted to visual inspection and instrumental analyses.

2.2. Visual Inspection

According to the WHO guidelines [2], we focused on differences in packaging, labeling, and physical appearance of the dosage forms characterized by specific size, shape and color in order to identify potential counterfeiting or deterioration. Suspected samples were further submitted to instrumental analysis to identify non-visual quality issues such as the presence of undeclared active ingredients or excipients, impurities and related contents.

2.3. Instrumental Analysis Methods

2.3.1. Reagents and Solutions

1) Reagents

Quinine sulfate (99.0%) and metronidazole (99.9%) reference substances were purchased from Fagron NV (Waregem, Belgium); Methanol LC grade from Avantor Performance Materials BV (Deventer, The Netherlands); hydrochloric acid (37%) and Ammonium formate (98.1%) from VWR International BVBA (Leuven, Belgium); Ultrapure water produced with Milli-Q Plus 185 water purification system (Millipore, Billerica, MA, USA); Dimethylsulfoxide “DMSO-d6” from Bruker (Billerica, MA, USA); Acetanilide and Sulfanilamide standards from ThermoQuest Italia S.p.A (Milan, Italy); Helium 6.0 (=99.9999 Vol%) and Oxygen 4.5 (=99.995 Vol%) from Alphagaz (Liège, Belgium).

2) Sample solutions for HPLC analysis

The test sample solutions for quinine tablets were prepared by dissolving in methanol LC grade accurately weighed quantities of the powders obtained from 20 tablets of each sample. Several dilutions were done to make final solutions equivalent to 100 µg/mL of quinine as analyte. The reference solutions were prepared in the same way to obtain the targeted 100 µg/mL concentration of the quinine. Two independent reference substances and three independent test sample solutions were prepared for the system suitability testing (SST) and for assay test.

On the other hand, metronidazole was involved in the analysis as a wrong active ingredient in quinine tablets, and preparation of the standard and assay test solutions was done as following:

Standard preparation: Dissolve in methanol LC grade an accurately weighed quantity of metronidazole CRS to obtain 200 µg/mL, and dilute half with the mixture water/methanol (80:20, v/v) to obtain a final standard solution of about 100 µg/mL. Two independent reference solutions were prepared.

Assay preparation: Dissolve in methanol LC grade a quantity equivalent to about the average weight of one tablet from 20 single dose units finely powdered; make appropriate dilutions in methanol to obtain about 200 µg/mL assuming that one tablet may contain 200 mg of metronidazole, filter each solution on a microporous membrane (preferably 0.45 µm pore size or less) and dilute as done for the standard solutions to have about 100 µg/mL of metronidazole.

3) Sample solutions for UV-Visible spectroscopic analysis

A fast UV identification test using a spectrophotometer was done on the following solutions:

Quinine sulfate reference solutions at 50 µg/mL and 100 µg/mL: Dissolve in methanol LC grade a quantity of quinine sulfate CRS in order to obtain approximately 200 µg/mL of stock solution. Then, make appropriate dilutions with water to obtain 50 and 100 µg/mL solutions.

Test sample solutions at 50 µg/mL and 100 µg/mL: Dissolve in methanol LC grade a portion of fine powdered quinine tablets in order to obtain a stock solution containing 200 µg/mL of quinine sulfate active ingredient. Then, make appropriate dilutions with water to obtain expected concentrations of 50 µg/mL and 100 µg/mL solutions.

Metronidazole reference solutions at 50 µg/mL and 100 µg/mL: Use metronidazole CRS and prepare as per quinine sulfate reference solutions.

Blank solutions: Prepare mixtures of methanol-water at two proportion levels (25:75, v/v) and (50:50, v/v) to simulate the blank conditions at 50 µg/mL and 100 µg/mL respectively.

2.3.2. HPLC Method

The analyses by HPLC were carried out on a Waters 2695 Alliance (Waters, Milford, MA, USA) separation module coupled to Waters 2996 photodiode array (PDA) detector (Waters) applying an in-house developed method for quinine analysis and a USP procedure for metronidazole in tablets as described below. The system is controlled with Empower 2.0 software (Waters).

1) HPLC operating conditions for quinine analysis as developed in-house

For quinine analysis, the chromatographic separation was done in a Zorbax SB-C8 (dp 3.5 µm) column (150 mm × 4.6 mm ID) maintained in a compartment thermostatted at 35°C, applying as mobile phase a isocratic mixture of methanol and 10 mM ammonium formate buffer adjusted to pH 2.5 with 6 N hydrochloric acid (40:60, v/v) that was pumped at 1 mL/min. The sample solutions, thermostatted at 15°C in a compartment, were introduced onto the separation system at a rate of 10 µL and monitored under the UV detection fixed at 230 nm.

2) HPLC operating conditions for metronidazole assay as wrong active ingredient in quinine tablets

For metronidazole analysis, chromatographic separation was done also with Zorbax SB-C8 (150 mm × 4.6 mm ID, dp: 3.5 µm) column in isocratic conditions, but using as mobile phase

a mixture of methanol and water (80:20, v/v). Flow rate, injection volume and UV detection were maintained identical as for quinine except thermostatted sample and column compartments that were set at 15°C and 25°C, respectively.

2.3.3. Raman Spectroscopy Method

Intact sample tablets vs. intact small quantities of reference materials were used. The samples were analyzed with LabRAM HR Evolution (Horiba scientific, Kyoto, Japan) instrument equipped with two-dimensional Newton 970 front-illuminated EMCCD detector (1600 × 200 pixel sensor) (Andor Technology Ltd.), Leica 50× Fluotar LWD objective and 785 nm laser with a power of 45 mW (XTRA II single frequency diode laser, Toptica Photonics AG). A 300 gr/mm grating was used to record the spectra in the spectral range of 3200 - 100 cm^{-1}. The confocal slit-hole was fixed at 200 µm. Each spectrum results of two acquisitions of 1 second. The spectra were collected with the LabSpec 6 (Horiba Scientific) software. Once acquired, the spectra were baseline corrected using the Asymmetric Least Squares (AsLS) algorithm with a λ value of 10^5 and a p-value of 10^{-3}. The baseline corrected spectra were then scaled between 1 and 0 and compared to the spectral database. All spectrum processing and correlation coefficient computations were performed using Matlab R2013a software (The Matworks, Natick, MA, USA) and in-house routine coding.

2.3.4. Mass Spectroscopy (ESI-MS)

Four different solutions of the powdered fake quinine tablets (two samples), quinine sulfate and metronidazole references were prepared at 10 µg/mL in a mixture of methanol and water (90:10, V/V), and analyzed by direct injection in the ES+/MS analytical conditions hereafter described.

The mass spectrometry on a triple quadripole instrument from Waters Corporation (Milford, MA, USA) was used in a positive electrospray ionization mode (ES+), in MS and MS/MS modes. MassLynx 4.1 software (Waters Corporation) was selected for instrument control, data acquisition and handling. ES+/MS analytical conditions were set at Capillary Voltage 2.5 kV, dessolvation temperature 200°C, Source Temperature 115°C, Multiplier 650 V.

2.3.5. Nuclear Magnetic Resonance (NMR) Spectroscopy

The 1H NMR spectra were measured on a Bruker Avance (500 MHz) spectrometer (Wissembourg, France) equipped with a 5 mm TCI cryoprobe. Tetramethylsilane (TMS) was used as an internal standard. Chemical shifts were reported in δ values (ppm) relative to internal TMS. The abbreviation s = singlet, d = doublet, t = triplet, m = multiplet and b = broad were used throughout, and data were processed using Topspin 2.1 software (Bruker, Wissembourg, France).

The reference solutions of quinine sulfate and metronidazole were prepared by dissolving 2 mg of the compound or equivalent of 2 mg for quinine powdered tablets in 700 µl of DMSO-d6 and then introduced into a 5 mm tube for analysis.

2.3.6. Elemental Analysis (CHNS)

Elemental analysis CHNS was also used to determine the percent weight of specific atoms of carbon (C), hydrogen (H), nitrogen (N) and sulfur (S) in the suspected organic compounds, and therefore enabling to verify their empirical formula (C x H y N j S k).

In this study, elemental analysis (CHNS) was realized on a Thermo Electron Corporation Flash EA 1112 CHNS Elemental Analyzer (Interscience sprl, Louvain-la-Neuve, Belgium) equipped with a dry sample autosampler on the top of furnace, and under Eager 300 dedicated software control for organic elemental analysis in the following analytical conditions: 900°C for sample combustion, 130 mL/min of the Carrier Flow, 100 mL/min for Reference Flow, 250 mL/min for Oxygen Flow, 5 sec for Oxygen Injection End (0 sec for Oxygen analysis), 12 sec for Sample Delay Time, 10 min for Run time, and 2 - 3 mg for Sample weight.

2.3.7. UV-Vis Spectroscopy

A UV spectrophotometric analysis was done using a CECIL, CE 2501 model (2000 series) spectrophotometer (Cecil Instruments Limited, Milton Technical Centre, Cambridge, UK) by recording and comparing the reference and sample test solutions' absorption spectra acquired in the range between 200 and 400 nm.

3. Results

3.1. Visual Inspection Findings

The pictures in **Figure 2** and **Figure 3** elucidate several cases of default and suspicious information confirming the usefulness of visual inspection at first line in decision making on drug quality when the product's physical appearance presents evident deviations to the quality standards. Indeed, various cases of poor quality medicines were detected such as the presence of discolored film coated quinine tablets, the packaging of artesunate powder for injection in different size boxes, the unlike batch numbers of artesunate powder for injection vials and outer packaging boxes, the fake packaging of artemether-lumefantrine blister tablets labelled COMBIART (Strides Arcolab Ltd) in LUMARTEM boxes (Quality Chemical Industries Ltd.), and infiltrated artemisinine-based combination therapy drug (ACT) from Uganda to Rwanda with a specific marginal note stating "Government of Uganda, For Public Use Only, Not for Sale". Note that the declared quinine tablets samples manufactured and/or distributed by HOLDEN MEDICAL (WEZEP-THE NETHERLANDS), HOLDEN MEDICAL (LELYSTAD-THE NETHERLANDS), and ELYS CHEMICALS (NAIROBI-KENYA) seemed to be visually normal. After these findings, the analyses continued following the step-by-step guide of **Figure 1**.

3.2. General Pharmacopeial Tests

As simple analytical tests, the following were processed: uniformity of mass, friability, disintegration, identification and assay according to the International Pharmacopoeia (4^{th} edition 2014) and United States Pharmacopoeia (USP 37-NF 32). The friability and disintegration tests results were within the specifications, but not the uniformity of mass for two particular samples manufactured and/or distributed by HOLDEN MEDICAL (WEZEP-THE NETHERLANDS), and ELYS CHEMICALS (NAIROBI-KENYA) illustrated in Appendix (**Figure A1** and **Figure A3**). The mass of the product supposed to be manufactured and/or distributed by HOLDEN MEDICAL (WEZEP-THE NETHERLANDS) was 292.4 ± 9.1 mg (average and standard deviation, n = 20 tablets).

The second quinine sample said (but not verified) to be manufactured by ELYS CHEMICALS (NAIROBI-KENYA) was found with similar average mass of 297.6 ± 6.1 mg

per tablet (n = 20 tablets). These results were surprising and not coherent since the two samples were declared to contain 300 mg of quinine sulfate. Hence, one can worry about the content of the active ingredient if the average weight is less than the declared content per tablet regardless the amount of excipients. Then, we decided on the basis of sampling periods and the incoherence of weight, to study and present these suspicious samples into two cases, one on underweight quinine tablets labelled HOLDEN MEDICAL (WEZEP-THE NETHERLANDS) and ELYS CHEMICALS (NAIROBI-KENYA), and another on the normal weight tablets of quinine labelled HOLDEN MEDICAL (LELYSTAD-THE NETHERLANDS).

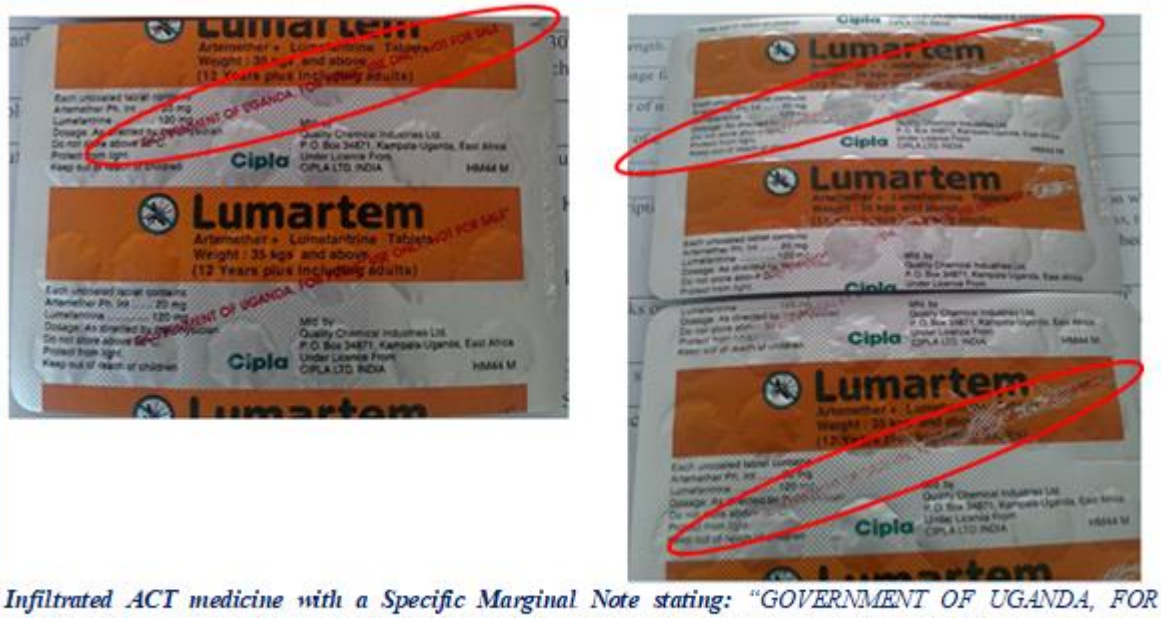
Discolored Film Coated Quinine Tablets

Different size boxes of Artesunate powder for injection

Mixed batch numbers: LA 120508 on the Box and ZA 120304 on the Vial (Artesunate powder for injection)

Figure 2. Photographs of poor quality quinine tablets and artesunate powder for injection.

Mixed manufacturers' products: ***COMBIART blister tablets*** *(Batch N° 7221596, Exp. Date: 04/2016, Mfg. by Strides Arcolab Limited / country not specified), registered in Nigeria under NAFDAC Reg. No: A4-6700 ;* ***LUMARTEM boxes*** *(Batch N° Q40037, Mfg. Date: 01/2014, Exp. Date: 12/2015, Mfg. by Quality Chemical Industries Ltd. Kampala-Uganda)*



Infiltrated ACT medicine with a Specific Marginal Note stating: *"GOVERNMENT OF UGANDA, FOR PUBLIC USE ONLY, NOT FOR SALE" Mfg. by Quality Chemical Industries Ltd. Kampala-Uganda) purchased in Rwanda. The second picture shows a sample of the same product but with intentional scratched marginal note to deceive the vigilance of consumers or inspectors.*

Figure 3. Photographs of Artemether-Lumefantrine combination medicine illicitly introduced to Rwanda.

3.3. First Case

3.3.1. Fluorescence Analysis

As a rapid identification test in the laboratory, we performed a visual observation of quinine fluorescence in both reference and sample solutions under UV light at 366 nm. It clearly showed noticeable difference between quinine sulfate reference at 10 µg/mL that was greenish-blue color fluorescence whereas the suspected sample solutions at the same concentration level did not present any fluorescence (**Figure 4**).

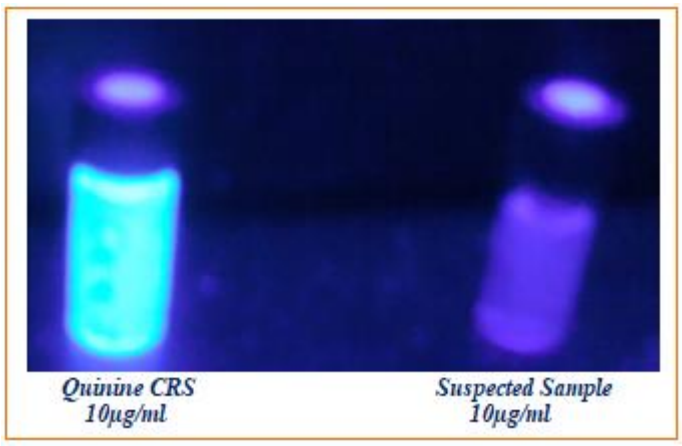


Figure 4. Quinine identification under UV light at 366 nm for fluorescence

3.3.2. HPLC Analysis

To explore the suspicious and illogical situation noticed above (Section 3.3.1), we carried out the most used separative technique in pharmaceutical analysis, namely HPLC. As shown on the chromatograms in **Figure 5**, the retention time of the major peak in the suspected false quinine tablets' solutions was 2.33 minutes, while the one of quinine reference substance eluted at 4.14 minutes. In addition, the analytical method was able to reveal two distinctive cinchona alkaloids at low concentrations generally present with quinine raw material or reference substance. The two alkaloids were identified cinchonidine and dihydroquinine eluting at 3.27 minutes and 5.29 minutes, respectively, and were not present in the suspected samples. Furthermore, the corresponding UV spectra extracted from PDA for the two suspected samples were totally different from the one for quinine reference substance (**Figure 5**), and the detected differences in terms of retention times and UV spectra were followed by

further analytical investigation to identify the unknown component in the two samples.

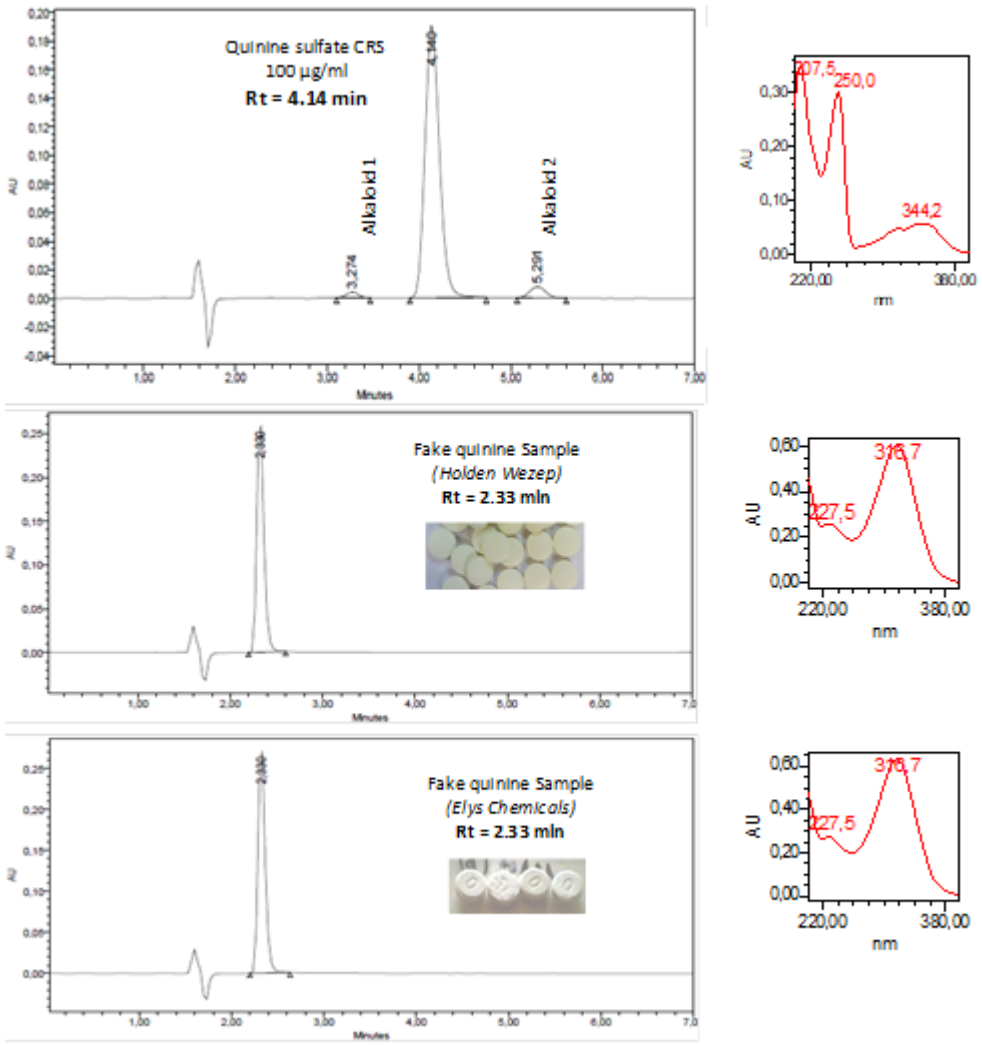


Figure 5. HPLC chromatograms and UV spectra of quinine sulfate CRS vs. Fake samples

3.3.3. Raman Spectroscopy

To pursue with the laboratory investigation, the Raman spectroscopic technique was used involving four basic steps to identify unknown components in the counterfeit products: 1) data acquisition on the studied samples, 2) instrument library search and update, 3) candidate products selection by fitting the experimental spectra to those in the database, and 4) identity confirmation of the unknown components by analyzing potential candidate substances vs. the samples.

The Raman spectra of the suspected quinine samples were found different from that of quinine sulfate reference substance. By comparing to Raman spectra database set up in the laboratory, it was noticed that the correlation coefficient corresponded very likely to metronidazole as potential candidate compound present in the suspected quinine tablets (cf. **Table 1** and **Figure 6** and **Figure 7**). Considering this new information, further analytical techniques were needed to confirm the identity of the unknown component.

Table 1. Raman spectrometry database search results for potential candidate products

Candidate product	Correlation coefficient
Methylprednisolone	-0.064
Metronidazole	**0.996**
Miconazole nitrate	-0.022
Microcristalline cellulose	0.109
Naphazoline HCl	0.161
Norfloxacine	0.111
Noscapine HCl	-0.026
Oxazepam	-0.034
Paracetamol	0.002
Paroxetine HCl. ½ H_2O	0.074
PEG 20000	-0.074
Phenazone	-0.074
Phenoxymethylpenicilline	-0.084
Phenylephrine HCl	-0.003
Pheniramine maleate	0.139
Piperacillin Na	0.038
Piperaquine	0.390
Povidone	-0.022
Prednisolone	-0.045
Proguanil HCl	-0.015
Propranolol HCl	0.265
Pseudoephedrine HCl	-0.012
Quinine 2HCl	***0.281***
Quinine sulfate	***0.140***
Saccharinate Na	-0.066

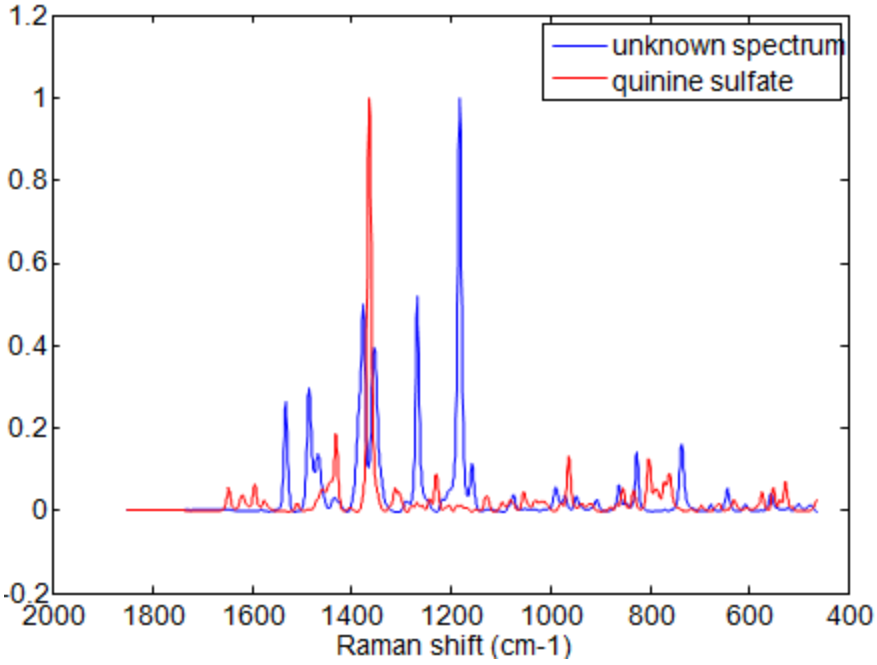


Figure 6. Raman spectra overlay for one of the two fake quinine tablets vs quinine sulfate CRS

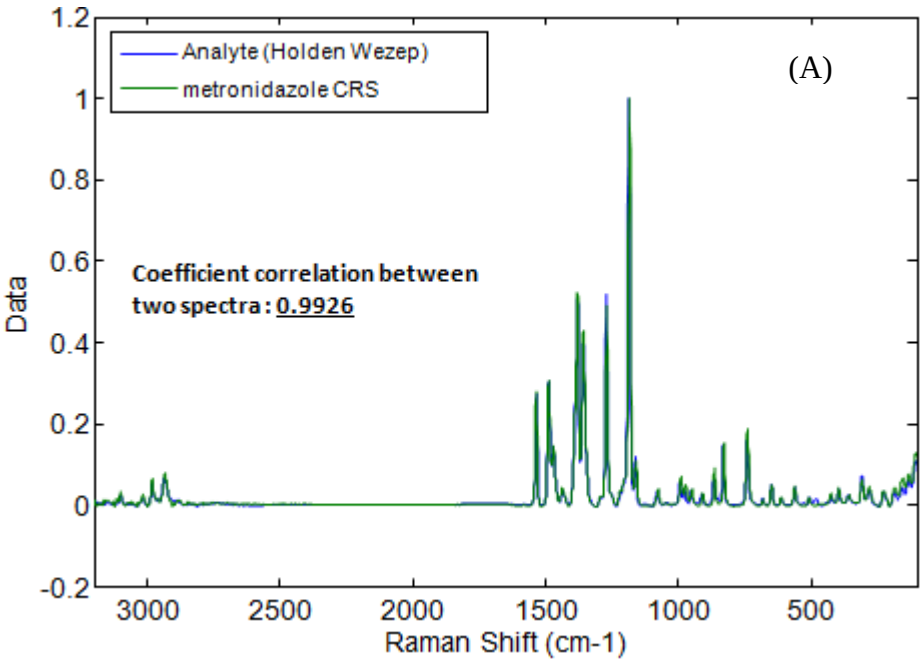


Figure 7. Raman spectra overlay for HOLDEN WEZEP fake quinine tablets vs metronidazole CRS

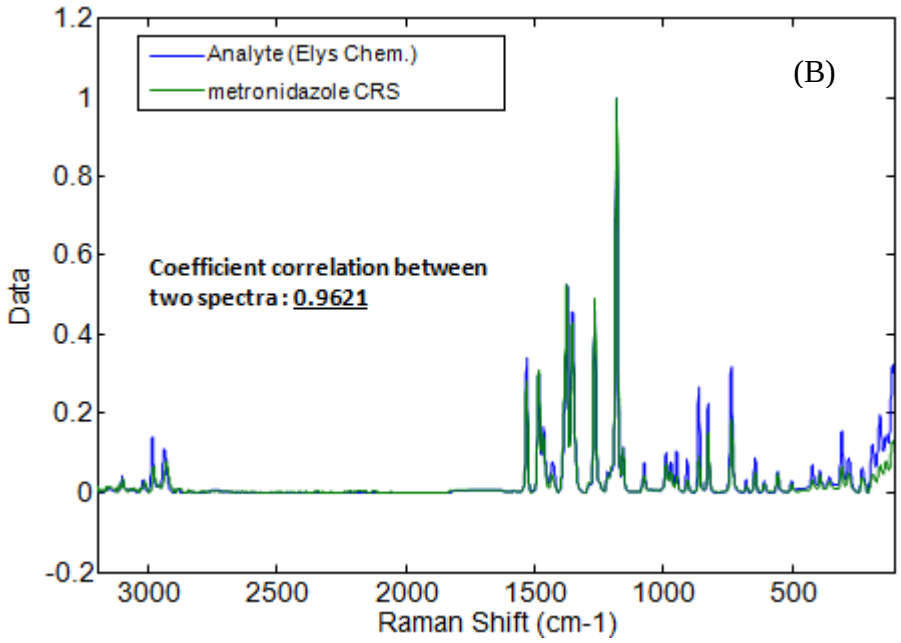


Figure 7. Raman spectra overlay for ELYS CHEMICALS fake quinine tablets vs metronidazole CRS

3.3.4. Mass Spectrometry

Both the MS and MS/MS were used. Their spectra presented in **Figure 8** and **Figure 9** confirmed the presence of metronidazole in the suspected quinine tablets. Indeed, the two samples have identical peaks with metronidazole reference substance, fragmentation profiles are very similar (172 > 128, 172 > 111) and relative abundance of the 128 and 111 peaks is the same in metronidazole CRS and in the two fake quinine samples.

3.3.5. Nuclear Magnetic Resonance (NMR)

By comparing respective 1H NMR spectra, all signals of the “fake quinine tablets” exactly matched with the signals of metronidazole CRS but not with quinine sulfate (**Figure 10**), confirming again the presence of metronidazole in the suspected quinine tablets. Based on these previous analytical results (HPLC, Raman and MS), one can already confirm the absence of the declared active ingredient in the two samples.

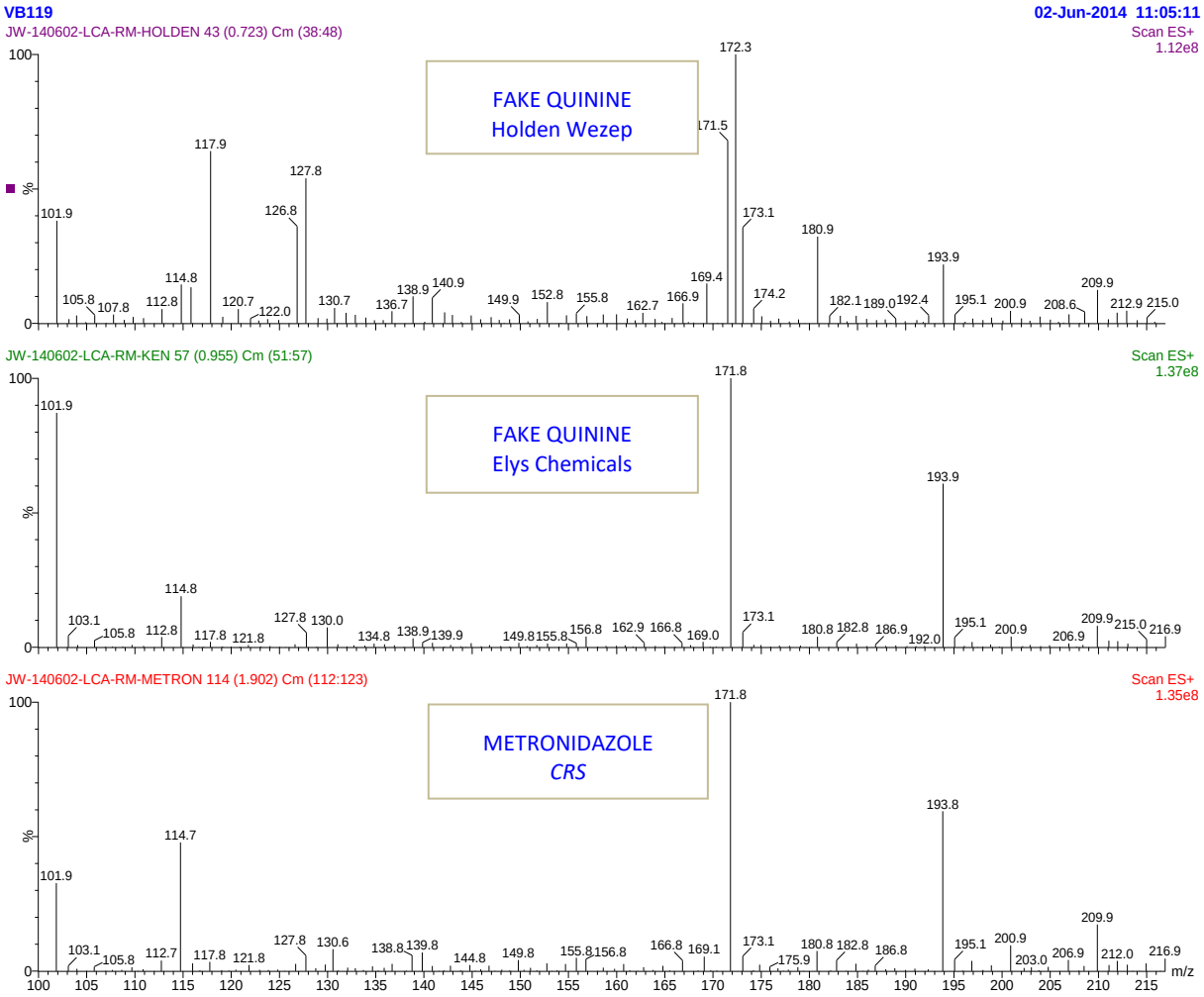


Figure 8. Comparison of ES+/MS spectra of metronidazole and the two fake quinine tablets (Elys chemicals and Holden Wezep) at 1.35e8, 1.37e8 and 1.12e8 Scan ES+ respectively.

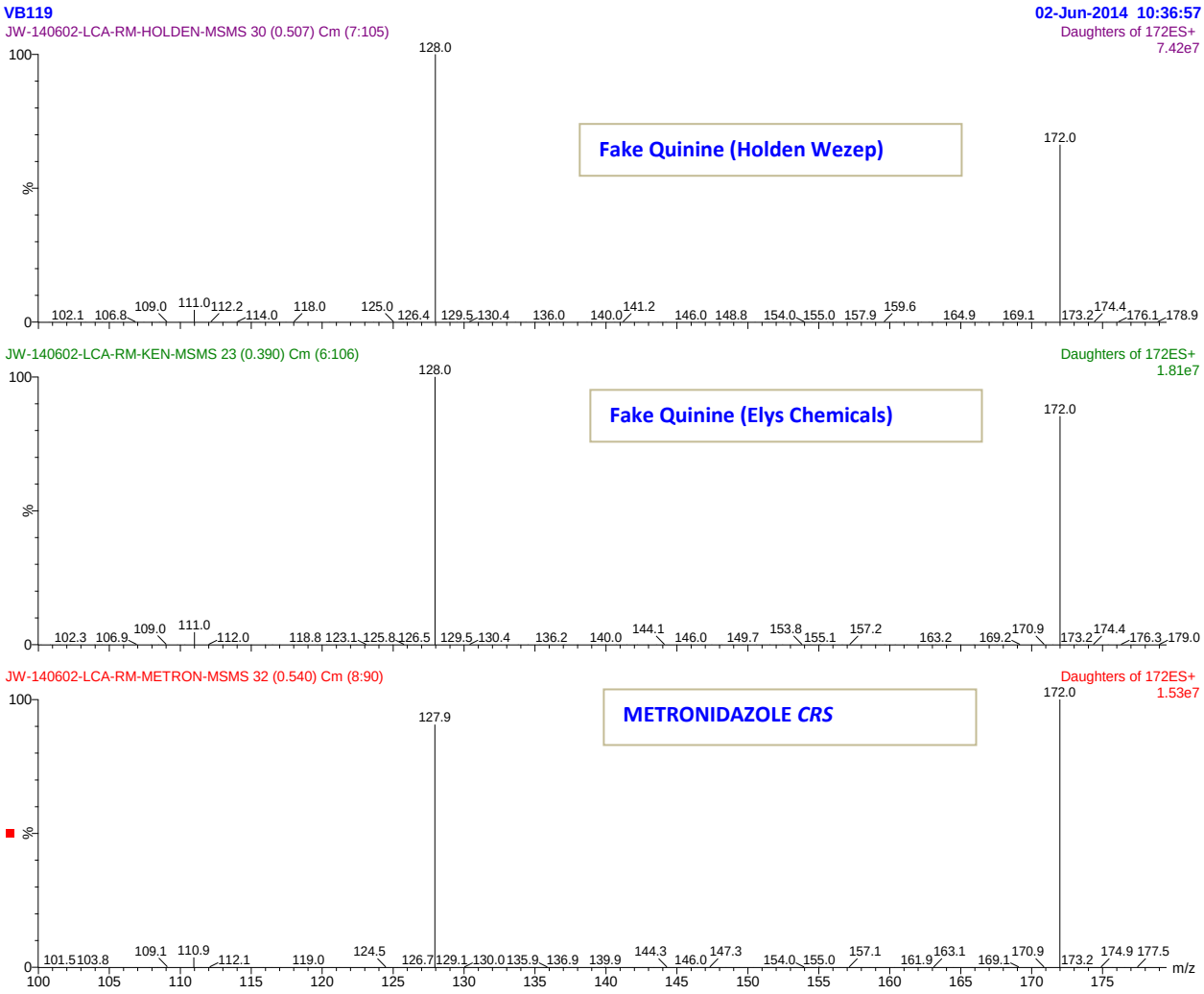


Figure 9. Comparison of ES+/MSMS daughters for metronidazole CRS, fake quinine (Elys chemicals) and Holden Wezep at 172 ES+1.53e7, 172 ES+1.81e7 and 172 ES+7.42e7 respectively.

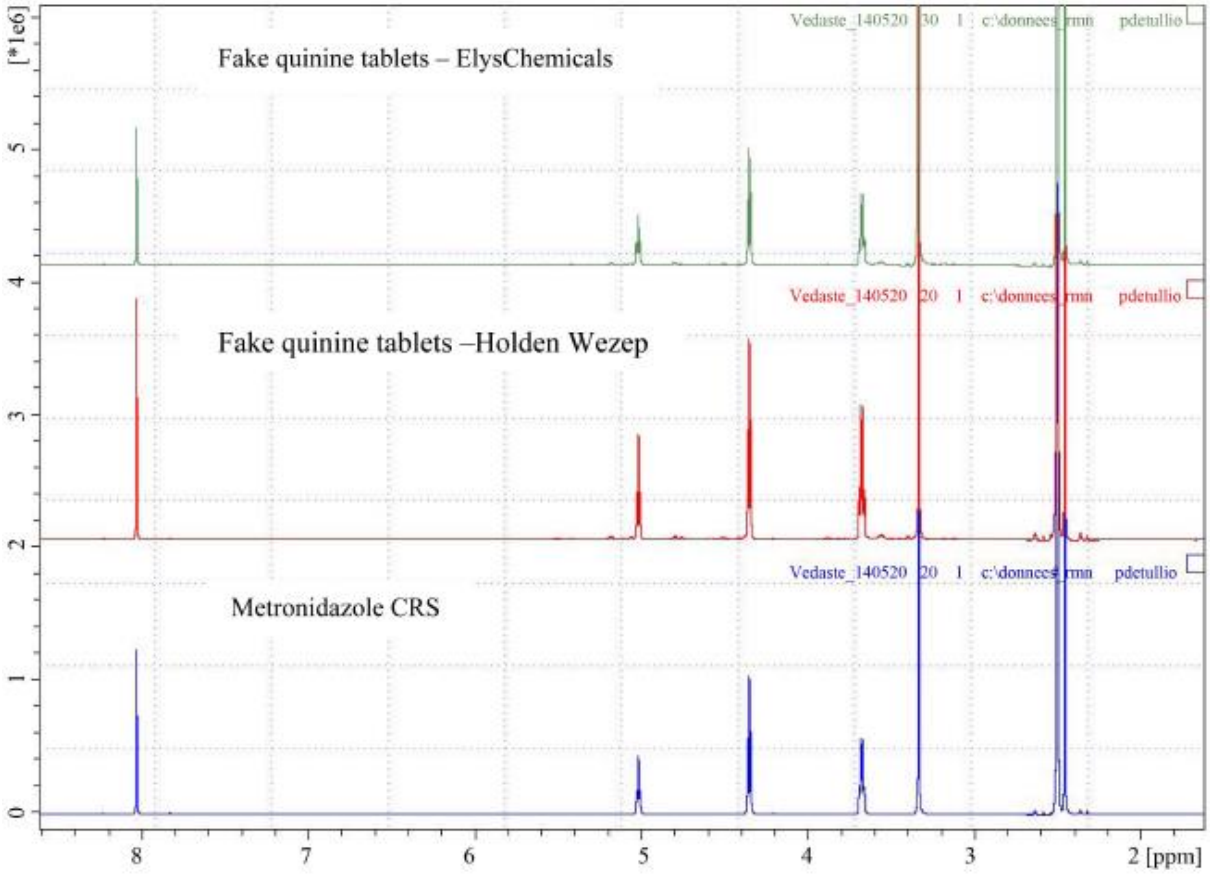


Figure 10. NMR spectra of fake quinine tablets and metronidazole reference substance

3.3.6. Elemental Analysis (CHNS)

Finally, the contents in (%) of carbon, hydrogen, nitrous and sulfur elements in the two samples were determined by elemental analysis. Considering ±0.4% - ±0.5% for carbon and nitrous, ±0.7% - ±0.8% for hydrogen, and ±1% for sulfur as calculated acceptable error limits to each considered element, one can notice that the two fake quinine tablets have totally different results from that of quinine sulfate CRS as can be seen in **Table 2**.

Indeed, the experimental % values obtained for N, C, H and S in the suspected quinine samples (Holden Wezep, and Elys Chemicals) are very closer among themselves, and closer to the experimental % values of metronidazole reference substance. However, they are particularly different from quinine sulfate for nitrous (about 3 times higher) and carbon (about 1.5 lower).

In addition, sulfur was not present in the two suspected and fake quinine samples. Such findings correspond more to metronidazole than to quinine sulfate. In fact, the absence of sulfur is not normal since quinine sulfate is used as raw material in the manufacture of quinine tablets and the sulfur content should be found in these samples.

Table 2. FlashEA 1112-CHN/S experimental results for metronidazole, quinine sulfate and fake quinine tablets.

Atom	**Metronidazole CRS**		**Sample 1:** Holden Wezep	**Sample 2:** Elys Chemicals	**Quinine sulfate CRS**	
	Theoritical (%)	Experimental (%) (n = 2) mean	Experimental (%) (n = 2) mean	Experimental (%) (n = 2) mean	Theoritical (%)	Experimental (%) (n = 2) mean
N	24.55	25.14	22.21	20.59	7.16	7.67
C	42.1	42.11	46.00	41.85	61.36	60.89
H	5.3	5.38	5.77	5.49	6.95	6.98
S	0.0	0.0	0.0	0.0	4.1	3.4

3.4. Assay Test Results of Metronidazole in the Two Fake Quinine Tablets

After characterization and identification of the unknown component in the two fake quinine tablets, we decided to determine the content of metronidazole in the two particular formulations. We used an adapted analytical method to the USP-37/NF-32 (2014) as described in Materials and Methods. The content of metronidazole in the first sample (HOLDEN MEDICAL, WEZEP-THE NETHERLANDS) was found 81.6%, and in the second sample 81.0% (labeled ELYS CHEMICALS NAIROBI-KENYA).

3.5. Second Case

Another case of suspected quinine sulfate tablets was discovered one year later after the first study case. The sample was labelled HOLDEN MEDICAL (LELYSTAD-THE NETHERLANDS) and immediate suspicion on this product quality was mainly based on the fact that the name “HOLDEN MEDICAL (THE NETHERLANDS)” already known from the previous analysis on another sample of quinine sulfate tablets labelled HOLDEN MEDICAL (WEZEP-THE NETHERLANDS) found containing metronidazole as active ingredient. The two types of HOLDEN MEDICAL quinine tablets samples are illustrated in pictures given in Appendix (**Figure A1** and **Figure A2**).

3.5.1. Visual Inspection and General Pharmacopeial Tests

Visual inspection and general pharmacopeial tests such as the uniformity of mass, friability and disintegration tests were processed as done in the first case analysis, but identification was done on a UV-Vis spectrophotometer to collect the UV spectra for comparison, and therefore confirm similarity with the first case. The assay test was not performed on this sample as the main purpose was to see if the product was also counterfeited with metronidazole as in the previous Holden sample despite their differences in shape, color, size and probably manufacturing sites (WEZEP-THE NETHERLANDS for the previous sample, and LELYSTAD-THE NETHERL-ANDS for the new sample). The new suspected quinine sulfate tablets 300 mg average weight was found 426.5 ± 3.6 mg which is logical including the excipients content. The tests of friability and of disintegration complied with the pharmacopeial specifications.

3.5.2. UV-Vis Spectroscopy

This technique was used to rapidly identify the active ingredient in that sample by comparing its UV spectrum to those of metronidazole and quinine sulfate reference substances. The UV spectrum of the sample did not match with the spectrum of quinine reference solution, however and again surprisingly the sample UV spectrum matched very well with metronidazole reference standard solution. After confirming that the new sample labelled HOLDEN MEDICAL (LELYSTAD-THE NETHERLANDS) contains the same wrong ingredient as in the HOLDEN MEDICAL (WEZEP-THE NETHERLANDS) sample, we decided to not perform further analysis with advanced analytical techniques as done in the first case, because the relevant information on the nature of counterfeiting was already known.

4. Discussion

During this study, careful visual inspection has enabled to detect easily a series of poor quality antimalarial medicines such as the presence of 1) discolored film coated quinine tablets that may contain toxic substances, impurities and or sub-degradation substances. The discoloration should be a result of poor storage conditions and subsequent degradation, poor manufacturing or even counterfeiting; 2) Different sized boxes and mixed batch materials of

artesunate powder for injection that may also be a result of drug counterfeiting or substandard manufacturing conditions; 3) Packed COMBIART blister tablets (Mfd. by Strides Arcolab Ltd) in LUMARTEM boxes (Mfd. by Quality Chemical Industries Ltd.) is clearly a result of drug counterfeiting because normally no official manufacturer should mix two different manufacturers' products; 4) Artemether-Lumefantrine combination tablets infiltrated to Rwanda with a marginal note stating "GOVERNMENT OF UGANDA, FOR PUBLIC USE ONLY, NOT FOR SALE" illegally introduced to Rwanda and sold. Another related quality issue to the later product, is that some illicit vendors tried to scratch the marginal note in order to cheat the vigilance of patients or inspectors.

On the other hand, laboratory testing had revealed surprising results on three different samples of tablets declared to contain quinine sulfate 300 mg and labeled HOLDEN MEDICAL (WEZEP-THE NETHERLANDS), HOLDEN MEDICAL (LELYSTAD-THE NETHERLANDS) and ELYS CHEMICALS (NAIROBI-KENYA).

Normally the average weight of tablets medicines is always greater than the declared content of active ingredient(s) due to the presence of excipients, however this was not the case for Holden Medical (Wezep-The Netherlands) and Elys Chemicals (Nairobi-Kenya) who have lower average weights than the declared active ingredient's amount. The identification test of quinine fluorescence under UV light had also failed on both samples, and further analysis with advanced analytical instruments such as HPLC, Raman spectroscopy, Mass spectroscopy, NMR and Elemental Analysis revealed that the two types of quinine tablets contain metronidazole instead of quinine. For Holden Medical (Lelystad-The Netherlands) quinine tablets samples of the second case, the average weight cannot cause suspicion to poor quality since it was higher than the declared active ingredient's amount. However, the UV spectrum has confirmed substitution of quinine sulfate by metronidazole. Something surprising is that the three fake quinine tablets despite their different shapes, color, and names (labelling) have the same wrong ingredient metronidazole which is an antimicrobial, antiprotozoal/anti-amoebic. On the other hand, this is very cunning from counterfeiters as metronidazole is relatively bitter like quinine, but more than ten times cheaper [21].

Quantitative analysis of metronidazole in HOLDEN MEDICAL (WEZEP-THE NETHERLANDS) and ELYS CHEMICALS (NAIROBI-KENYA) quinine tablets revealed that not only counterfeiters have used a wrong active ingredient, but also the products are

under-doses. This may refer to thinking about falsification of the active ingredient during production or falsification during packaging and labelling. Hence, several public health consequences may be possible for consumers such as the failure in malaria treatment, development of drug resistance for plasmodium, increase of morbidity and mortality due to malaria, etc.

After noticing that the products were counterfeit and therefore dangerous to the public health, immediate alerts were sent to the vendors, the Pharmacy Task Force of the Ministry of Health (Rwanda), and to the National Procurement and Distribution Center for appropriate measures against these products; but also for careful control of other medicines that would be circulating in the country from the same manufacturers or distributors.

Concerning the other antimalarial samples, even if visual inspection was not satisfying, the declared active ingredients were confirmed to be present at the expected and declared content. However, note that each medicine should comply with all required quality specifications for safe use.

From this study, one can notice how advanced analytical techniques such as HPLC, Raman spectroscopy, mass spectroscopy, NMR, and elemental analysis (CHNS) are complementary and efficient in the detection and characterization of unknown components in counterfeit medicines. One can also rely on the database of simple analytical technique such as the UV-Vis spectroscopy that was successfully used to detect the second case of suspected Holden quinine sample.

5. Conclusion

A strategic approach to detect poor quality medicines and identify unknown components in counterfeit antimalarial medicines has been applied in this study that allowed detection of several cases. Some of them were detected by means of simple and affordable tools such as visual inspection and tablets weighing, whereas in other cases it was necessary to exploit complex tools namely pharmacotechnical and analytical ones. The latter were applied for deep analyses in order to evaluate the quality of three quinine sulfate tablets samples that were found containing metronidazole instead of quinine. Timely alerts were sent to all concerned parties, medical and pharmaceutical, based on scientific evidences found for

appropriate measures against the spread of the detected harmful products. In fine, the study results illustrated and highlighted the necessity to strengthen National Medicines Regulatory Authorities, especially in developing countries in order to ensure among other responsibilities that all distributed medicines are of the required quality, safety and efficacy.

Acknowledgements

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APPENDIX

Figure A1. Picture of HOLDEN MEDICAL (WEZEP-THE NETHERLANDS) fake quinine sample. **(a)** Sample labeling information on a "cubical plastic box"; **(b)** Packed biconvex yellow tablets without a break line; **(c)** Logo of either the manufacturer or distributor (not specified!); **(d)** Logo of HOLDEN MEDICAL (picked from internet for comparison: http://www.holdenmedical.com/web/show/contact), last visited on Sept 12th, 2014.

Figure A2. Picture of HOLDEN MEDICAL (LELYSTAD-THE NETHERLANDS) fake quinine tablets sample. **(a)** Sample labeling information on a "cylindrical plastic box"; **(b)** Packed white flat-faced bevel-edged bisect tablets; **(c)** Logo of either the manufacturer or distributor (not specified!).

Figure A3. Picture of ELYS CHEMICALS (NAIROBI-KENYA) fake quinine tablets sample. Note: The picture was taken after the friability test.

PART V - GENERAL DISCUSSION

This thesis was made possible conducted under the methodology described in Chapter III, and the following key activities were performed:

V.1 Selection of therapeutic classes for study

Different sources of information on the use and management of medicines mainly in Rwanda were exploited to know which therapeutic classes are mostly used and which medicines are widely consumed in order to select the ones that our thesis had to focus on.

We consulted the Pharmacy Department of the Ministry of Health, and pharmacists working in the private sector and public institutions who kindly shared different lists of medicines they use to supply their clients. In addition, other useful information were obtained from different reports published in the websites of the Rwandan government and international health-related organizations such as the World Health Organization, the Global Fund to fight AIDS, tuberculosis and malaria, the Management Sciences for Health, the Bill & Melinda Gates Foundation, the Clinton Health Access Initiative, the Médecins Sans Frontières, etc. On this basis, antiretroviral and antimalarial medicines were obviously the most appropriate candidates for our study due to their wide use and high risk to counterfeiting. The targeted molecules are also mentioned in the national lists of essential medicines or other medicines officially authorized in Rwanda (cf. [16-18] on p. 78).

V.2 Selection of analytical technique

Normally medicines and related raw materials have dedicated analytical methods. It should be noted that in some circumstances there is need to combine several techniques in order to assess the identity and assay of a given sample in complicated situations such as complex samples, and counterfeit medicines whose real composition is never known either qualitatively or quantitatively before testing.

The choice of HPLC technique was very helpful due to its very high sensitivity, selectivity and accuracy. It is also a powerful analytical technique for simultaneous analysis of several compounds in samples, widely recommended in pharmacopoeias, well known in different laboratories across the world, and whose equipment are available in Rwanda. Kovacs et al.

[30] (cf. p. 31) have also confirmed that HPLC could be considered as first choice analytical technique in drug analysis in our countries.

V.3 Methods development

The new methods were developed thanks to the Design of experiments – Design space (DoE-DS) methodology explained in *Part I.3* (cf. p. 23) for the methods that imply simultaneous analysis of several compounds such as the screening methods.

DoE methodology is a systematic approach used to investigate the factors effects and interactions between them by modifying multiple factors at time. It is time and cost efficient methodology. Then, DSs defined as "multidimensional combination and interaction of input variables that have been demonstrated to provide assurance of quality" are computed from the collected data of applied experimental designs. It should be noted that DoE is needed to define DS and that performing analysis within DS is not considered as a change, thus DoE-DS methodology ensures the robustness of the developed methods, that is an another advantage in the frame of geographic method transfer.

To realize our DoE, the studied key variables (analytical factors or parameters) were pH of the aqueous mobile phase, temperature (T°) and gradient time (T_G) as basically having very significant impact on the chromatographic behavior and that need to be optimized for better chromatographic results.

As mobile phase, methanol and volatile buffer salts (ammonium formate, ammonium acetate, and ammonium bicarbonate) were mainly privileged for affordability and effectiveness in yielding good chromatographic results. Moreover, the volatile buffering salts present an advantage of being transferable to LC/MS systems for further analysis (if needed) after DAD detection. Then, classic reversed phase (C8 and C18) analytical columns were also privileged for their wide use in LC analysis, and availability on the market. In fact, by applying HPLC coupled with UV-Vis detector or DAD, we have assurance to identify a very wide range of basic, acidic, and neutral substances that possess chromophoric groups. This was demonstrated in *Sections 1 and 2* of Results while checking the capability of the screening method of antiretroviral and antimalarial medicines, respectively. We extended the

methodology application in detecting other substances that are not expected to be in the original composition of medicines such as in case of counterfeiting or substandard, or withdrawal medicines in Rwanda. 11 antimalarial molecules were concerned as well as 14 out of 15 NSAIs and 13 antibiotics. They were traced in the developed methods. Two counterfeit cases of fake quinine tablets and fake Coartem (artemether/lumefantrine) tablets were detected during sample analysis and well described in *Sections 4 and 5* of Results.

Sometimes there are useful analytical methods that can be developed without much effort, in particular for the analysis of few molecules in a sample. In this case, we used a simple methodological approach of simple adjustment or adaptation of preexisting methods related to the studied analytes. We were able to optimize and improve the working analytical conditions up to their suitability that were observed with desired separation results in few experiments without need of long and relatively complex design of experiments.

V.4 Methods validation

As explained in Part I.3 (cf. p. 23-24), methods validation is a requirement to guarantee the reliability of future analytical results, and we used the recommended ICH Q2R1 guidelines complemented by the Total Error strategy. The latter is a very practical statistical tool that helps assessing quickly and visually the fitness or ineptness of the methods to their intended use by comparing the experimental results with the required acceptable limits plotted in the accuracy profiles drawn from the validation results.

Prior to the validation step, the development was extremely useful through DoE-DS methodology to demonstrate the selectivity of the methods considering the separation of analytes. Also, the pre-validation was carried out by assessing any interference checked with the chromatograms of sample, the reference substances, and the blank solutions. The retention times and UV spectra were taken into account.

Method trueness was found very good for the two validated methods with relative bias values less than 2% to the targeted sample concentrations (100%) as well as the method precision for which the RSD values were less than 2.5% for both repeatability and intermediate precisions. These two validation parameters were obtained thanks to the accuracy of results as can be

observed in the accuracy profiles illustrated in *Sections 1 and 3*. Their tolerance intervals were included within the predefined acceptance limits of 5% and 10% according to the type of sample. The methods presented good linearity (with coefficient of determination ($R^2 > 0.99$) and adequate slope and intercept.

Method for quantitation of high amount does not need determining the LOD and LOQ. However, since we expected low dosage or contamination in the frame of counterfeit, falsification or degradation, these two parameters were determined. The LOQ (lower and upper) allowed us to fix the dosing range of the validated methods (for artesunate powder analysis, and for ARV tri-therapy (emtricitabine / tenofovir disoproxyl fumarate / efavirenz) in tablets.

V. 5 Methods transfer

We performed geometric transfers from long to shorter analytical columns in order to improve the sample analysis throughput by reducing the analysis time, and the volume of rejected wastes. It is also very important not only to keep efficient analytical methods, but to have fast ones especially while analyzing poor quality medicines or suspected ones in order to know as soon as possible if the analyzed samples comply with the required quality standards or not and therefore take appropriate measures as soon as possible in protecting the users.

Then beside those geometric transfers, some methods have been successfully transferred from the developer laboratory in Belgium to Rwanda, and currently they are being used in routine analysis at the Laboratory of Analysis of Foodstuff, Medicines, Water and Toxicants (LADAMET / University of Rwanda) and at Rwanda Biomedical Center / Medical procurement and Production Division (RBC/MPPD). An extension of the methods transfer to other laboratories such as the National Quality Control Laboratory of medicines based at the Rwanda Standards Board (RSB) is also planned. All the developed methods shall serve a lot in the analysis of different antimalarial medicines and ARVs used in Rwanda.

V.6 Sample collection and application of the methods

Antimalarial medicines were the most concerned by sampling because ARVs are generally procured from WHO prequalified manufacturers under strong quality assurance policies set by the key international donors that support and control more severely their procurement and distribution.

Strategic sampling sites were selected in towns, suburbs, and country borders near Burundi, Tanzania, Uganda and the D.R. of Congo in order to collect samples from potential outlets such as pharmacies, hospitals, health centers, and tentatively informal supply channels that may be found during the sampling.

Actually, the Rwandan Ministry of Health has the reputation to close down any pharmaceutical establishment that does not comply with the regulation in force, and in general there is no informal market of medicines in Rwanda except rare cases that may pass unnoticed. Then, the sampling technique was random sampling by considering different batch numbers, manufacturers, strength, and dosage forms (tablets, capsules, injectables, etc.).

On the other hand, there are other samples of antimalarial medicines that were collected from Kinshasa (DRC) and Cotonou (Benin) in the frame of scientific collaboration, and they were added to the samples from Rwanda in order to assess their quality, and thus know whether they present potential public health risks if poor quality is discovered among them.

V.6.1 Antiretroviral medicines

Two fixed-dose combinations of emtricitabine, tenofovir disoproxyl fumarate and efavirenz, were collected from Rwanda and Benin. One of the two samples was composed by the three APIs, and the second by two.

As presented in *Section 1*, the developed method helped to identify the three APIs (sample coded A), but unfortunately the content for two (emtricitabine and tenofovir disoproxil fumarate) failed to comply with the specifications (90.0 % - 110.0 %) at 80 ± 1 % and 85 ± 1 %, respectively. However, the content of those APIs in the other sample coded B, were

acceptable (93 ± 2% and 91 ± 1%), respectively. We realized that sample A had expired few months ago, and assume that this might be in relation with the loss of 15% or more on assay. In the other sample, even if results are acceptable, they are also nearer to 90%.

V.6.2 Antimalarial medicines

As presented in *Sections 3 to 5*, different samples of antimalarial medicines collected from Rwanda, DRC and Benin were successfully analyzed thanks to the developed and validated methods in this thesis, taking mainly advantage of their character of being simple and fast.

In *Section 3*, three samples of Artesunate powder for injection sampled in Rwanda gave very satisfactory results at the level of 99 to 100% of the claimed nominal content. *In Section 4*, on the analysis of 9 samples of artemether / lumefantrine tablets sampled in the DRC and 13 samples of the same Artemisinin-based combination therapy medicine sampled in Rwanda in very different generic formulations, 3 of the 9 samples from the DRC were found at risk of out-of-specifications either for underdose cases where one sample presented a result of 90.7 ± 1.8% for artemether content, or for overdose cases where two samples presented 108.4 ± 1.8 % and 109.4 ± 1.8 % for lumefantrine content. The other samples gave very satisfactory results comprised between the acceptance limits (90.0 % - 110.0 %), and public health authorities should enforce quality assurance and quality control policies to protect the population against poor quality medicines dangers. All 13 samples from Rwanda were found compliant with the specifications, but always attention and enforcement of QAQC policies are highly needed to prevent all medicines from counterfeiting or substandard. On the other hand however, the suspected batch of Coartem from Benin for being counterfeit was confirmed not containing any of the two expected active ingredients, and this is a very serious concern to the public health that needs national and international collaborations in controlling the supply chain of medicines and protecting the population against all dangers that may rise from the use of poor quality medications. Another successful application of these methods is presented in *Section 5* where two types of quinine tablets sampled in Rwanda were detected not containing the declared active ingredient, but contained another substance that was later discovered to be metronidazole thanks to the use of other powerful analytical techniques. This case is also very alarming as the one of fake Coartem sampled in Benin, and it reveals the

gravity of the phenomenon of drug counterfeiting that must be fought severely by all, worldwide and jointly.

V.7 Application of advanced techniques

As aforementioned, among the analyzed samples were two types of Quinine sulfate 300 mg sampled in Rwanda whose one of them had a very abnormal average weight inferior to the declared content in quinine as active ingredient, and one sample of artemether / lumefantrine from Benin suspected to be counterfeit that were submitted to more complex analytical techniques in order to identify the nature of unknown compounds in their composition. Hence, the Raman spectroscopy, mass spectroscopy, NMR spectroscopy, and elemental analysis were used to supplement HPLC results and other basic tests carried out earlier.

As described Chapter I, Raman spectroscopy is a vibrational spectroscopic technique capable of providing qualitative and quantitative information on the active pharmaceutical ingredients as well as on the composition of the sample matrices (excipients) that can be present in a variety of pharmaceutical formulations. This technique has advantages of being non-destructive, fast, requiring little or no sample preparation, and it is environmental friendly. On the other hand, Mass spectrometry (MS) is a powerful analytical technique used to: (i) quantify known materials, (ii) identify unknown compounds within a sample, and (iii) elucidate the structure and chemical properties of different molecules with very high specificity. The complete process involves the conversion of the sample into gaseous ions, with or without fragmentation, which are then characterized by their mass-to-charge ratios (m/z) and relative abundances. Then, NMR which is another powerful analytical technique used in determining chemical molecular structures and capable of providing both qualitative and quantitative information on the studied analytes was used to complement the results obtained with the other techniques.

These advanced analytical techniques were used to supplement each other, and they gave very conclusive results on the type of unknown compound in the two fake quinine samples, as well as the absence of the two declared active ingredients in the sample expected to contain artemether - lumefantrine.

However, note that it is not mandatory to use several advanced analytical techniques as long as one or two of them can give satisfactory information to know the real identity of the unknown compound(s). Note also that the identification of unknown compounds can be fast if there are rich spectral libraries to consult and compare with the unknown sample results, but it is always necessary to close the testing with the analysis of the sample vs the reference substance of the product revealed to be the unknown analyte, and confirm the similarity.

V.8 Results reporting

Finally, when the quality control is finished and that the results are ready, they should be communicated as soon as possible to all concerned persons and to health authorities whenever necessary to allow taking appropriate measures timely, especially in case of poor quality products that must be recalled for destruction. Normally, the responsible health authorities use rapid alerts by media (radio, TV, SMS, internet, etc.) in order to spread the information to the largest number of the population.

For example, the detected fake quinine tablets that contained metronidazole instead of quinine were communicated to the Pharmacy Department of the Ministry of Health in Rwanda and to the national store of medicines (RBC/MPPD) to allow them knowing the problem and do the necessary actions accordingly. They were also informed about the fake Coartem product not containing any of the declared active ingredients in order to check whether it has not been supplied in the country even though the samples was collected from Benin.

Our scientific collaborators in the DRC and Benin, were also informed as soon as the alarming results were confirmed on the fake medicines, in order to inform the competent authorities in their countries for appropriate measures timely.

PART VI – GENERAL CONCLUSION AND PERSPECTIVES

VI.1 General Conclusion

Since one of the major problems of counterfeit medicines is in direct relationship with the health of the population, the main purpose of this thesis was to contribute to the protection of public health in Rwanda.

This main objective was split into four specific objectives carried out through our work and based on two pharmacological classes of medicines, the antiretroviral (ARV) and antimalarials. Those are vital medicines used in developing countries in the treatment of the two pandemic diseases, HIV and malaria, for which any lack of active ingredient, below or overdoses, substitution or degradation, will have a direct negative impact on health of population.

On basis of method lifecycle strategy, the first part of our contribution was devoted to the development of reliable generic analytical methods for the screening of the two groups of medicines cited above, which for remind are largely used in Rwanda.

In this way, we successfully developed generic LC methods for analysis of 18 ARVs (namely the ABC, ddI, EFV, FTC, IDV, 3TC, LPV, NFV, NVP, RTV, SQV, d4T, TDF, AZT, RAL, ATZ, DRV, ETV) and 4 major excipients (NpG, NpS, BHA and BHT) by applying DoE/DS methodology. At first 15 of them and 4 excipients were selected for optimization using D-optimal design involving column temperature, gradient time and pH of buffer solution as factors under investigation. Only the two later factors had significant effects on peak separations within the investigated domain. At the end of Monte Carlo simulations, this screening method proved to be able to separate simultaneous analysis of 19 compounds using as mobile phase a mixture of methanol and 10 mM ammonium hydrogen carbonate buffer. In the same configuration, we developed other methods for the analysis of ARVs in solid and liquid dosage forms.

Always in the same context, another of our contribution was the construction of DoE-DS knowledge domain for ARVs. Indeed, this tool is very useful since one can use it in the prediction of other optimal analytical conditions without performing any other experiments, for example the case for the analysis of any other fixed-dose combination or mono-

component of ARV active ingredient with one or more of the four studied excipients. This part is described in the section 1 of Results.

Concerning the antimalarial medicines, we exploited a DoE-DS knowledge domain previously elaborated by previous PhD-students in the Laboratory of Pharmaceutical Analytical Chemistry. Twelve compounds including eight active ingredients (arteether, artemether, artesunate, atovaquone, lumefantrine, mefloquine, proguanil, quinine) and four excipients (BHA, BHT, nipagin and nipasol) were able to be separated by an overall screening method besides a sub-screening method for the analysis of quinine, artemether and lumefantrine in liquid dosage forms containing the four excipients, and a generic sub-screening method for the analysis of quinine, arteether, artemether, and artesunate in injectable forms. This part was developed in the section 2 of Results.

In section 3 of the Results, we also developed simple isocratic methods thanks to methods adaptation or adjustments approach. Taking into account the advantages of isocratic methods, we were able to set up three generic methods for the analysis of (i) artesunate, arteether and artemether in injectable forms; (ii) artemether and lumefantrine in tablets; and (iii) quinine-resorcin with major cinchona alkaloids (cinchonine, cinchonidine, quinidine, and dihydroquinine).

To complete this part of the present thesis, the ability of the developed methods to quantify the targeted molecules with accuracy in routine was then demonstrated. By means of the total error strategy, we successfully performed the validation of the method for the combination of FTC/TDF/EFV in a fixed dose tablet (see section 1 of Results) that was applied in assay of those ARVs on real samples. In section 3 of the Results, we also presented the validation of the method for analysis of artesunate powder for injection as a new antimalarial medicine. We obtained an adequate accuracy profile clearly demonstrating its suitability according to its intended use.

The third aspect of our contribution was devoted to the detection of several cases of counterfeiting. In the section 4 of Results, we showed how a counterfeit artemether / lumefantrine medicine was detected from people complaints after analysis with simple tests, namely the thin layer chromatography and disintegration test. Thanks to Raman spectroscopy and HPLC, this falsification hypothesis was confirmed. All these tests established the

unfortunate case of absence of both active ingredients in the sample collected from Benin, and the same batch was found in Gabon, Mali and Cameroun which means infiltration in different countries.

Finally, in the section 5, we proposed a strategic approach to detect poor quality medicines. Stepwise, again counterfeit antimalarial medicines were detected by means of simple and affordable tools such as visual inspection and tablets weighing, followed by complex tools namely pharmacotechnique and analytical ones. The quality of three quinine sulfate tablets samples were found containing metronidazole instead of quinine. Through the network, timely alerts were sent to all concerned parties.

VI.2 Perspectives

In future prospects, we plan to:

- Complete the validation of the remaining developed methods before their use in routine analysis. We expect to do that locally in Rwanda, that will need to ensure adequacy of the obtained validation results through the training of analysts, and the qualification of available equipment;

- Complete the transfer of the remaining methods from the University of Liège (Belgium) to Rwanda for efficient use in the quality control of antimalarial medicines and antiretrovirals (ARVs), especially in the frame of the analysis of suspected counterfeit/falsification batches. This will be an extremely important support to the health institutions concerned by HIV and malaria treatments;

- Conduct as much as possible a large study on the quality assessment of different antimalarial medicines and ARVs used in Rwanda in both public and private sector, considering different aspects that can affect the quality of a medicine such as manufacturers (generics vs brand products), storage conditions, dosage forms, strength, etc. This kind of information is currently lacking in Rwanda and these studies will be conducted in close collaboration with the Ministry of Health.

PART VII – SUMMARY OF THE THESIS

English version

This thesis is part of the fight against poor quality medicines and especially their spread in developing countries. Its main goal is to contribute to the protection of public health in Rwanda and neighboring countries given the danger that this type of medicines represents to the health of the population. This contribution focuses on the development of reliable and inexpensive analytical methods that can be used in Rwanda. The technique of high performance liquid chromatography has been selected driven by the availability of LC equipment at the local level.

Two therapeutic classes have been selected, namely antiretrovirals and antimalarials. They are among the medicines whose pathologies are classified in the TOP 10 causes of death and disability in Rwanda, so very widely used and therefore potentially targeted by counterfeiters. Two approaches of methods development have been applied. A first more complex but rich in analytical information (retention times, UV-Vis spectra, light absorption and intensity of peaks, sensitivity to parameter variations, etc.) that required modeling and statistical processing based on the experimental designs whose results make possible to determine the design spaces in which the analytical parameters can vary without altering the quality of the expected results. Another simple approach was followed for the development of simple methods in isocratic mode from other methods that must be adjusted to obtain good results on the separation of the targeted analytes. This has been applied for antimalarials. One part of the the methods was validated using the total error strategy that includes the accuracy profile as a decision tool for compliance.

Moreover, in support of HPLC, other orthogonal analytical techniques were used to confirm the cases of counterfeit samples. These are mainly the Raman spectroscopy, mass spectroscopy, and nuclear magnetic resonance.

Thus in the **Article 1**, a general screening method for simultaneous analysis of 15 ARVs and 4 major excipients was optimized on a C18 column; 100 x 4.6 mm, 3.5 µm (dp); a generic screening method for the analysis of ARVs in liquid dosage forms; and another method of analysis for solid dosage forms have been developed and optimized. Subsequently, a formulation of emtricitabine (FTC), tenofovir disoproxil fumarate (TDF) and efavirenz (EFV)

tablets was selected and fully validated under the analytical conditions of the method for solid dosage forms. Thanks to these methods, it is easy to detect counterfeits involving one or more of the studied analytes, but also other types of non-compliance such as overdoses or underdosages can be detected.

In the **Article 2**, two general screening methods for simultaneous analysis of 8 antimalarial medicines used in Rwanda and 4 major excipients were developed and optimized on a C18 column, 250 x 4.6 mm, 5 μm (dp) at the analysis times of 100 and 65 minutes per injection, and we preferred the shorter of the two methods (the one at 65 minutes) which was then geometrically transferred to the columns of 150 mm, 100 mm, and 50 mm of length in order to obtain shorter analysis times: 41.5 minutes on the 150 mm column, 23.3 minutes on the 100 mm column, and 11 minutes on the 50 mm long column.

In the **Article 3**, it is about simple development of methods through the adaptation or adjustment of other methods on less complex samples containing few analytes. In addition to this approach, another strategy based on sequentially followed experiments according to the obtained results has been applied, and we have developed three simple and rapid isocratic methods for the analysis of antimalarial medicines in less than 10 minutes by injection, or even less than 5 minutes.

The **Articles 4 and 5** deal with the applications of already developed methods. The **Article 4** is about an application of the method for analysis of artemether/lumefantrine in various generic forms commonly marketed in Benin, the Democratic Republic of Congo and Rwanda in tablets forms. The first part of analysis was done in Benin using conventional techniques based on thin layer chromatography, disintegration and uniformity of mass tests. Subsequently, a sample was suspected to be counterfeit due to lack of characteristic spots of both analytes and abnormal disintegration time of more than 3 hours without effect. This sample was analyzed with other artemether/lumefantrine tablets collected in the DRC and Rwanda, and HPLC test results revealed that the Beninese sample was a fake medicine containing neither artemether nor lumefantrine! This counterfeit has been confirmed by Raman spectroscopy. As for **Article 5**, we had a case of samples of quinine tablets whose HPLC results revealed the absence of quinine as the declared active ingredient, but the substitution with another compound which was later elucidated with advanced spectroscopic

techniques namely the Raman, NMR and mass spectrometry. The substituting molecule was surprisingly the metronidazole !

Version en français

La présente thèse s'inscrit dans le cadre de la lutte contre les médicaments de qualité inférieure et plus spécialement leur propagation dans les pays en développement. Elle vise comme but principal de contribuer à la protection de la santé publique au Rwanda et les pays voisins étant donné le danger que ce genre de médicament représente sur la santé des populations. Cette contribution est centrée sur le développement de méthodes analytiques fiables, peu coûteuses, utilisables au Rwanda. La technique de la chromatographie liquide à haute performance a été sélectionnée vue la disponibilité d'équipements au niveau local.

Deux classes thérapeutiques ont été sélectionnées à savoir les antirétroviraux et les antipaludéens. Elles font partie des médicaments dont les pathologies sont classées dans les TOP 10 des causes de décès et d'invalidité au Rwanda, donc très largement utilisés et par conséquent potentiellement ciblés par les contrefacteurs. Deux approches de développement des méthodes ont été appliquées. Une première plus complexe mais riche en information analytiques (temps de rétention, spectre UV-Vis, absorption de la lumière et intensité des pics, sensibilité des analytes aux variations des paramètres, etc.) qui a nécessité des modélisations et traitements statistiques basée sur les plans d'expériences dont les résultats permettent de déterminer les espaces de conceptions au seins desquels les paramètres analytiques peuvent varier sans altérer la qualité des résultats attendus. Une autre approche simple a été suivie pour le développement des méthodes simples en mode isocratique à partir d'autres méthodes qu'il faut ajuster pour obtenir de bons résultats sur la séparation des analytes étudiés. Celle-ci a été appliquée pour les antipaludéens. Une partie des méthodes a été validée au moyen de la stratégie de l'erreur totale qui inclue le profil d'exactitude comme outil de décision quant à la conformité.

Par ailleurs, en appui à la CLHP, d'autres techniques analytiques orthogonales ont été mise à contribution dans le but de confirmer les cas des échantillons révélés contrefaits. Il s'agit principalement de la spectroscopie Raman, la spectroscopie de masse, et la résonance magnétique nucléaire.

Ainsi dans l'**Article 1**, une méthode générale de criblage pour l'analyse simultanée de 15 ARVs et 4 excipients majeurs a été optimisée sur une colonne de type C18 ; 100 x 4.6 mm, 3.5 µm (dp) ; une méthode générique de criblage pour l'analyse des ARVs en forme

liquide ; et une autre méthode d'analyse pour les formes solides ont été développées et optimisées. Par après, une formulation d'emtricitabine (FTC), tenofovir disoproxil fumarate (TDF) et efavirenz (EFV) comprimés a été sélectionnée et entièrement validée dans les conditions analytiques de la méthode pour les formes solides. Grâce à ces méthodes, on peut facilement détecter des contrefaçons impliquant un ou plusieurs des analytes étudiés, mais aussi on peut détecter d'autre types de non-conformité comme les surdosages ou sous-dosages.

Dans l'**Article 2**, deux méthodes générales de criblage pour l'analyse simultanée de 8 antipaludéens utilisés au Rwanda et 4 excipients majeurs ont été mises au point et optimisées sur une colonne de type C18, 250 x 4.6 mm, 5 µm (dp) aux temps d'analyse de 100 et 65 minutes par injection. Nous avons privilégié la plus courte des deux méthodes (celle à 65 minutes) qui a été ensuite transférée géométriquement aux colonnes de 150 mm, 100 mm, et 50 mm de longueur afin d'obtenir des temps d'analyse plus courts : 41.5 minutes sur la colonne de 150 mm, 23.3 minutes sur la colonne de 100 mm, et 11 minutes sur la colonne de 50 mm de longueur.

Dans l'**Article 3**, il s'agissant d'une simple mise au point des méthodes rendues possibles grâce l'approche d'adaptation ou ajustement d'autres méthodes sur les échantillons moins complexes contenant peu d'analytes. En plus de cette approche, une autre stratégie basée sur les tests séquentiellement agencés les uns après les autres selon les résultats obtenus a été appliquée, et nous avons développé trois méthodes d'analyse en mode isocratique simples et rapides pour l'analyse des médicaments contre la malaria en moins de 10 minutes par injection, voire même moins de 5 minutes.

Quant aux **Articles 4 et 5**, ils portent sur des applications réelles des méthodes déjà développées. L'Article 4 est une application de la méthode d'analyse d'artémether – luméfantrine dans diverses formes de génériques couramment commercialisés au Bénin, au République Démocratique du Congo (RDC) et au Rwanda sous forme des comprimés. Une partie des échantillons était d'abord analysée au Bénin à l'aide des techniques classiques basée sur la chromatographie sur couche mince, et des tests de désintégration et uniformité de masse. Par après, un échantillon a été suspecté contrefait suite au manque des spots caractéristiques des deux analytes et au temps de désintégration anormal de plus de 3 heures sans effet. Cet échantillon a été analysé avec d'autres échantillons d'artémether –

luméfantrine comprimés collectés en RDC et au Rwanda, et les résultats d'analyse par CLHP ont révélé que l'échantillon du Bénin était un faux médicament ne contenant ni artémether ni luméfantrine ! Cette contrefaçon a été confirmée par la spectroscopie Raman. Quant à l'**Article 5**, nous avions un cas des échantillons de quinine comprimés dont les résultats HPLC révélaient l'absence de quinine en tant que principe actif déclaré, mais la substitution avec un autre composé qui a été élucidé après à l'aide des techniques spectroscopiques avancées dont le Raman, la RMN et la spectrométrie de masse. La molécule de substitution était étonnamment le métronidazole !

PART VIII - PUBLICATIONS

VIII.1 ARTICLES

[1] **V. Habyalimana**, J. K. Mbinze, A. L. Yemoa, P. Lebrun, E. Ziemons, J-L. K. Ntokamunda, P. Hubert, R. D. Marini, Optimization of LC Methods Using DoE-DS Database and Geometric Transfer of Methods for Rapid Screening of Poor Quality Antimalarial Medicines in Rwanda. Under development for submission to the International Journal of Science and Engineering Investigations (IJSEI)

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[4] **V. Habyalimana**, J. K. Mbinze, A. L. Yemoa, C. Waffo, T. Diallo, N. K. Tshilombo, J-L. K. Ntokamunda, P. Lebrun, P. Hubert, R. D. Marini, Application of Design Space Optimization Strategy to the Development of LC Methods for Simultaneous Analysis of 18 Antiretroviral Medicines and 4 Major Excipients Used in Various Pharmaceutical Formulations, J. Pharm. Biomed. Anal. 139 (**2017**) 8-21. https://doi.org/10.1016/j.jpba.2017.02.040

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