Assessment of Counterfeit and Substandard Antimalarial Medicines using High Performance Thin Layer Chromatography and High Performance Liquid Chromatography

DISSERTATION

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Dedication

This thesis is dedicated to my late father Hebron N. Mwalwisi who passed away in my absence, wife Nzota Victoria and our children Jestina Sharon, Lizbert Mimi and Jesse Gambares.

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List of abbreviations

API	Active Pharmaceutical Ingredient
B. P.	British Pharmacopoeia
EDQM	European Directorate for the Quality of Medicines and HealthCare
GMP	Good Manufacturing Practices
HPTLC	High-Performance Thin Layer Chromatography
IPC	In-Process Control
KAAD	Catholic Academic Exchange Service
LF-NMR	Low-Field NMR spectroscopy
LOF	Lack-of- Fit Test
MEDS	Mission for Essential Drugs and Supplies
NMRA	National Medicine Regulatory Authority
OMCL	Official Medicines Control Laboratory
Ph. Eur.	European Pharmacopoeia
Ph. Int.	International Pharmacopoeia
PMS	Post Marketing Surveillance
QC	Quality Control
RP-HPLC	Reversed-Phase High-Performance Liquid Chromatography
TFDA	Tanzania Food & Drugs Authority
TLC	Thin Layer Chromatography
UPLC	Ultra Performance Liquid Chromatography
USP	United States Pharmacopoeia
WHO	World Health Organization

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1. General Introduction

1.1 Etiology of Malaria

Malaria is a parasitic disease caused by different species of *Plasmodia*. The parasites include *P. falciparum* (common in tropical and subtropical areas), *P. vivax* (Asia, Latin America, and some parts of Africa), *P. ovale* (West Africa and the Western Pacific Islands), and *P. malariae* (worldwide) [1, 2]. These three important species can be differentiated from each other by the onset of malaria symptoms such as fever, headache, chills and vomiting. This takes 12-14, 10-20 and 30 days for *P. falciparum, P. vivax, and P. malariae,* respectively. *P. falciparum* is the most dangerous among these parasites with a complex life cycle (**Figure 1**) alternating between an asexual cycle in the body of female *Anopheles* mosquitoes (sporogony) and an asexual cycle in vertebrate hosts, which occurs in the body of an infected person (schizogony).

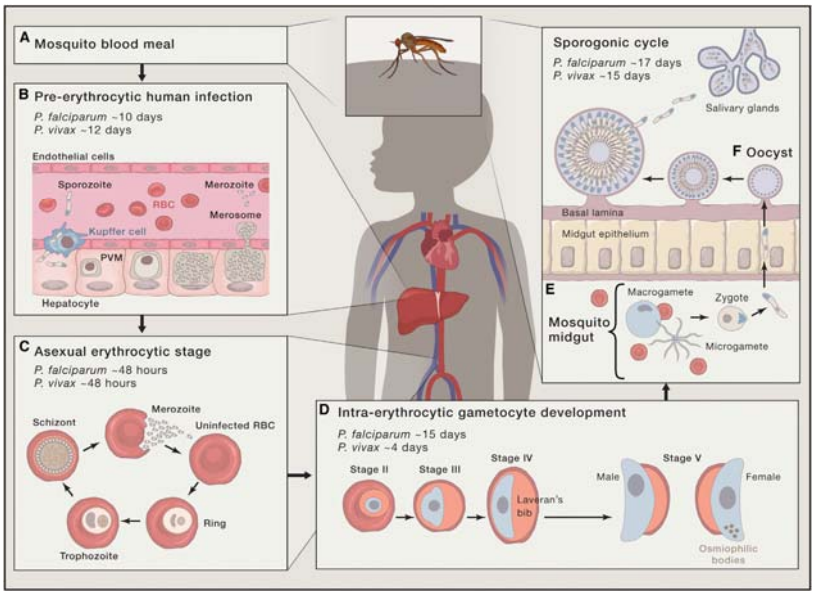

Figure 1: Life cycle of *P. falciparum*[1]

The sporozoites that were formed in the blood of female mosquitoes (from male and female hematocytes) are transmitted into the human body through a mosquito bit. The sporozoites enter the liver cells where they form primary tissue schizonts, which grow, divide, and

[1] reprinted with permission from Kevin Marsh, Centre for Tropical Medicine and Global Health, Nuffield Department of Medicine, University of Oxford, Headington, Oxford OX3 7BN, UK

transform into merozoites. The merozoites then enter the blood circulation of the person and diffuse into erythrocytes, where they develop further into maturity. The schizonts again divide and transform into merozoites, are periodically being released from the occupied cells, and attack a new group of erythrocytes, starting the cycle over. This process lasts for three or four days. The destruction of erythrocytes and the simultaneous entry of a large amount of merozoites in the blood are expressed by an onset of malaria fever, i.e. the perierythrocytic form of malaria which is not present in tropical countries. Other plasmodia species such as *P. malariae* and *P. ovale* have a different pathway of development being called exoerythrocytic form of malaria in which parasites in the merozoites stage of development remain in or enter the liver again. This restarts the erythrocytic cycle of development of plasmodia and the onset of relapse [1, 3].

Malaria remains a disease of global health importance [3] and a leading killer disease in tropical countries, particularly in sub-Saharan Africa [4-6]. About 3.3 billion people are at risk of suffering from malaria, whereby Africa accounted for over 80% and 90% of all malaria cases and deaths, respectively, in 2015 [3, 7, 8].

1.2 The antimalarial therapy

Medicines used in the treatment and prevention of malaria are classified into three groups based on the specific stage of the disease where the drug is effective, i.e. those that have an effect on the erythrocyte stage of life cycle, those that destroy the exoerythrocytic (or hepatic) stage, and those that affect both stages simultaneously (**Table 1**) [1, 9]**.**

Table 1: Medicines used in the treatment of malaria

Stage of plasmodial infection	**Groups and example of drugs**
Erythrocytic stage	4-Methanoquinolines (quinine and mefloquine)
	Phenanthrene (halofantrine)
	4-Aminoquinolines (chloroquine and amodiaquine)
	Sesquiterpene lactones (the artemisinin group including artesunate, artemether, and dihydroartemisinin)
	Dichlorobenzylidines (lumefantrine) which supplement the effectiveness of sesquiterpene
	Tetracyclines (doxycycline and tetracycline)
Exoerythrocytic (hepatic)	8-Aminoquinolines (primaquine and tafenoquine)
Both hepatic & erythrocytic	Biguanides (proguanil and chlorproguanil)
	Diaminopyrimidines (pyrimethamine)
	Sulfonamides (sulfadoxine and sulfalene) which potentiate the effectiveness of pyrimethamine

Quinine and its analog mefloquine belong to the 4-methanoquinoline chemical group (**Figure 2**). Quinine is a methoxylated derivative of cinchonine and the levorotatory isomer of quinidine. It consists of a quinoline ring at its fourth position which is bounded by a hydroxyl methylene bridge to a quinuclidine moiety. Quinine has been used as a base structure for the synthesis of copious compounds with antimalarial activity. Mefloquine contains a piperidine fragment instead of quinuclidine at C_4 of the quinoline ring while positions C_2 and C_8 are substituted with trifluoromethyl groups. Their mechanism of action is similar to that of chloroquine though believed to be of inferior activity. They also suppress large portions of the enzymatic system and therefore are being characterized as a general protoplasmid toxin which rationalizes the action of quinine on membranes, its local anesthetizing and its cardiodepressive effects [1, 10].

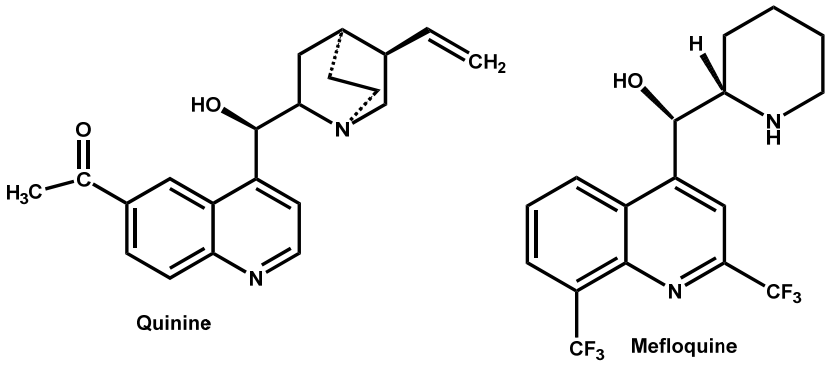

Figure 2: 4-Methanoquinoline group

Chloroquine and amodiaquine belong to the chemical group of 4-aminoquinolines and they are structurally related to pamaquine and primaquine (8-aminoquinolines) (**Figure 3a-b**). Chloroquine is currently seldomly used in malaria treatment due to parasite resistance [10-12]. The mechanism of action of chloroquine and its analogs is unknown but it is believed to inhibit the synthesis of nucleic acids of the parasite by intercalating their molecules between the orderly arranged base pairs into the spirals of the DNA of the parasite and thus preventing transcription and translation which significantly limits the synthesis of DNA and RNA in the parasite [1, 10].

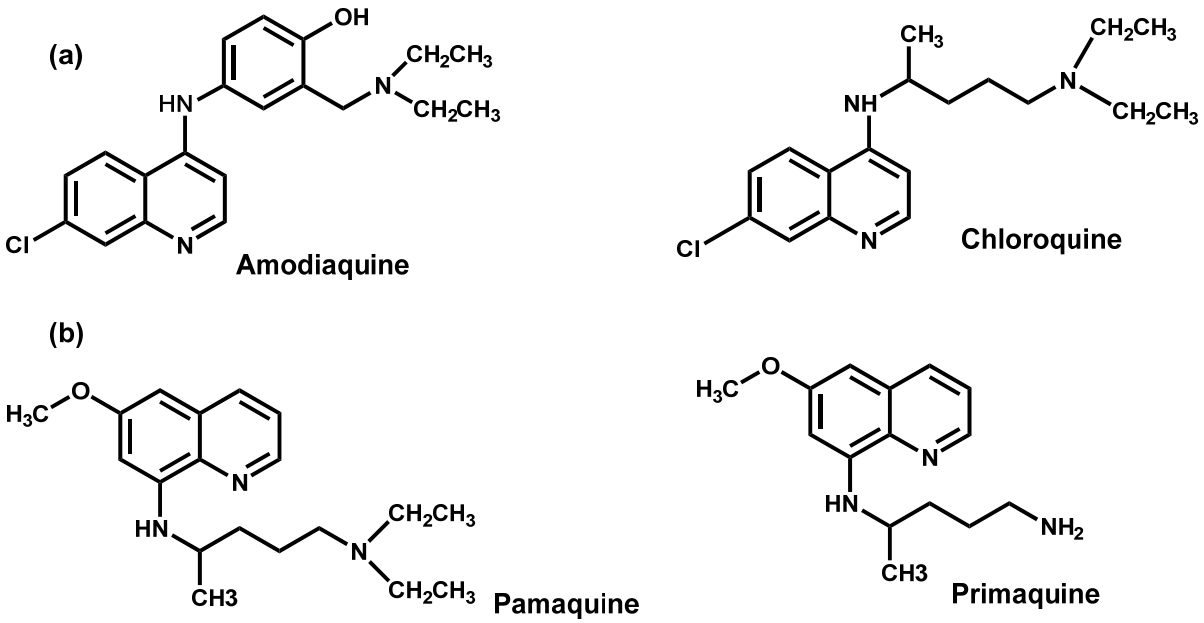

Figure 3: (a) 4-Aminoquinoline group and (b) 8-Aminoquinoline group.

Apart from activity against malaria parasite, chloroquine posses amoebicidal, immunosuppressive and antiarrhythmic properties [1]. Unlike the 4-aminoquinolines, primaquine and pamaquine activity are limited to tissue forms of the parasite in mammals and mosquitoes making them valuable drugs as they allow radical recovery and simultaneous prevention which is not achieved by using erythrocyte drugs. They act by interfering with the process of electron transfer in mitochondria of the parasite, causing damage to mitochondria enzymatic systems [1, 10].

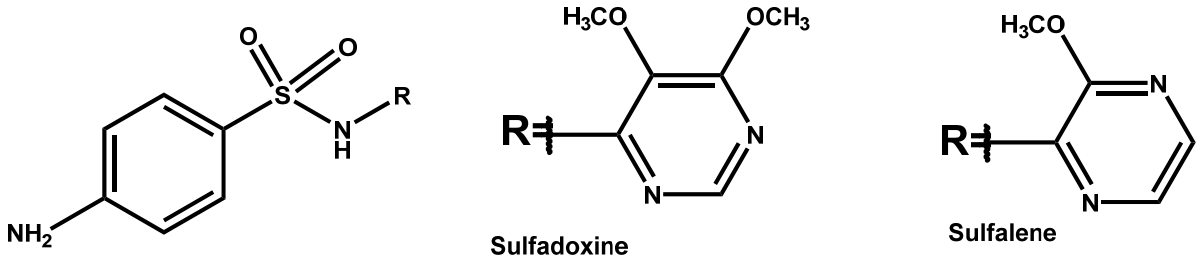

Figure 4: Sulfonamide group

Sulfadoxine and sulfalene are sulfonamides (**Figure 4**), well known antimalarial drugs which potentiate the effect of pyrimethamine (a diaminopyrimidine, cf **Figure 5b** in the inhibition of the dihydrofolate reductase in the malaria parasite [10, 13]. Due to parasite resistance, the combination of pyrimethamine with either sulfadoxine or sulfalene is no longer recommended for the treatment of malaria though it is still used in the prevention of malaria

in pregnant women [10, 11]. Pyrimethamine in combination with sulfadiazine or trisulfapyrimidine is indicated for the treatment of toxoplasmosis while sulfadoxine is also used for the treatment of bacterial infections of respiratory organs, the gastric and urinary tract, of osteomyelitis, sinusitis, and other infections [1].

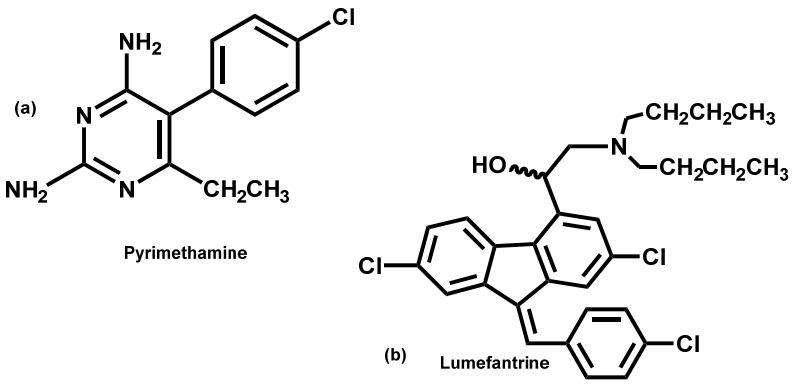

Figure 5: (a) diaminopyrimidine and (b) Dichlorobenzylidines group

Lumefantrine is a dichlorobenzylidine (**Figure 5b**) which is structurally related to halofantrine (a phenanthrene derivative, **Figure 6**). Lumefantrine has shown to be capable of acting synergistically with artemether (see below) in the treatment of uncomplicated *P. falciparum* malaria. Despite its safety records, there are concerns about its possible cross resistance with mefloquine which need to be addressed to maintain the therapeutic potential of this combination. Halofantrine is another therapeutic agent which is active against chloroquine-resistant *Plasmodium* parasites which is limitedly used due to cardiotoxicity. Nevertheless, their *N*-desbutyl derivatives are equally potent and devoid of cardiotoxicity in the case of halofantrine [10].

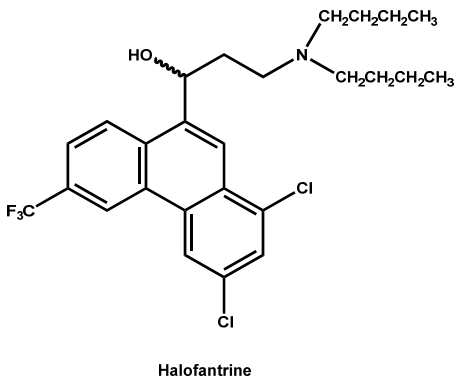

Figure 6: Phenanthrene group

Artemether belongs to the chemical group of sesquiterpenes; it is a relatively new, effective, well-tolerated drug which has been discovered and isolated from *Artemisia annua*. Although the exact mechanism of action of artemether and its derivatives (**see Figure 7**) is not certainly known yet, a cation-mediated formation of reactive intermediates and the reduction of the peroxide bridge are anticipated, resulting in an initial large reduction of parasite biomass. The remaining viable parasites can be removed by the less active but more slowly eliminated lumefantrine [2, 5, 9]. Both drugs are commercially available as fixed dose combinations.

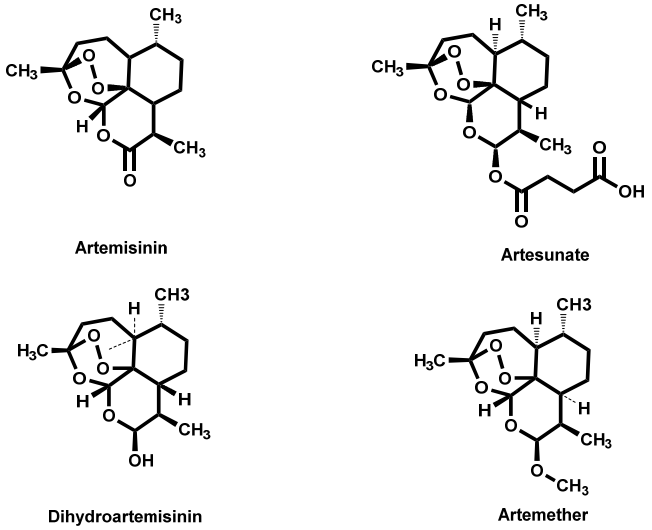

Figure 7: Sesquiterpene lactone group

The World Health Organization (WHO) recommends artemisinin-based combination therapies (ACTs) involving artemether and lumefantrine, artesunate and amodiaquine, artesunate and mefloquine, dihydroartemisinin and piperaquine, or artesunate and sulfadoxine/pyrimethamine (SP) for the treatment of uncomplicated *P. falciparum* infection in children and adults [2]. Citing Tanzania as an example, a combination of artemether-lumefantrine (ALu) is recommended at the country level as first-line therapy for uncomplicated malaria while quinine is the second line drug. Quinine also remains the drug of choice for the treatment of severe malaria [11, 14].

In malaria endemic areas, the WHO recommends that all pregnant women in their first and second trimester should be subjected to intermittent preventive treatment utilizing

sulfadoxine/pyrimethamine as part of antenatal care. Dosing is required to start in the second trimester and given at least one month apart, ensuring that at least three doses are received. Infants living in moderate-to-high malaria transmission should also receive intermittent preventive treatment with SP during a second and third round of vaccinations against diphtheria, tetanus, and measles. However, this applies only to areas where SP is still effective [2, 11, 12].

1.3 Problems facing malaria treatment

The fight for the eradication of malaria faces several problems whereby parasite resistance to commonly applied drugs is the most challenging one [5, 15]. This has forced a change in malaria chemotherapy paradigms as antimalarial drugs are no longer effective as they used to be in the past. In Tanzania for example, the Ministry of Health changed its malaria treatment policy from chloroquine to sulfadoxine/pyrimethamine (SP) monotherapy in August 2001 due to reported chloroquine resistances. However, it had to be changed again to highly efficacious artemisinin-based combinations four years later because of similar problems with sulfadoxine/pyrimethamine [11]. Under- or over-dosing contributed by an irrational use of medicines and the distribution of substandard or counterfeit medicines is among the reasons purported for the formation of parasite resistance [16-23].

1.4 Magnitude of substandard and counterfeit medicines worldwide

The quality of antimalarials is critical to the success of any treatment regimen as it determines its efficacy and safety. It is alleged that administering medicines of assured quality contributes in slowing down the development of resistances to antimalarial drugs. Therefore, the effort against the spreading and use of substandard/counterfeit medicines is equally important [6, 24]. Some reports suggest that up to 25% of medicines consumed in developing countries are counterfeited while recent studies performed by the WHO revealed that up to two-thirds of the samples collected in sub-Saharan African countries were substandard [6, 8, 25, 26]. National Drug Authorities (NDAs) are obliged to ensure the quality of medicines circulating in their respective markets through quality assurance programs. Routine quality testing and post-marketing surveillance (PMS) following

compendial protocols are among the strategies employed [8]. However, the implementation of these strategies in resource-constrained countries is hampered by a lack of resources required for operating the quality control laboratories in terms of consumables, chemicals, reference standards, equipment requiring specialized conditions such as air conditioning and regular maintenance, and skilled personnel. On the other hand, protocols and monographs used in the routine quality control testing and post-marketing surveillance of medicines also face several limitations [27], e.g. limited applicability or the necessity of expensive and delicate reagents.

1.5 Analytical methods for assessing the quality of medicines

The Minilab® which has been developed by the Global Pharma Health Fund is very useful for the field assessment of the presence of labeled active ingredients by using color reactions and thin layer chromatography (TLC) [28]. However, neither the content, nor organic impurities, nor other than the labeled drug can be identified and quantified accordingly. This means that it is difficult to detect counterfeits containing only trace amounts of an Active Pharmaceutical Ingredient (API) or an API substitute.

The internationally recognized pharmacopoeias such as the European Pharmacopoeia (Ph. Eur.), the British Pharmacopoeia (B. P.,), the United States Pharmacopoeia (USP), the Japanese Pharmacopoeia (J. P.,), and the International Pharmacopoeia (Ph. Int), provide monographs for the quality assessment of physical and chemical parameters of finished pharmaceutical products [29-33]. The tests and assays listed in pharmacopeias are restricted to a certain synthesis pathway or production procedure which means that the listed impurities are mostly starting material or side products from the synthesis pathway used. If a different route was applied, another impurity profile with deviating related compounds occurs. These impurities cannot be detected and determined by the tests prescribed in the monograph because a separation of the “new” impurities from the main compound and from each other is not guaranteed utilizing these methods. Moreover, none of the aforementioned pharmacopoeias is intended for the enlightenment of deliberate counterfeits. Taken together, currently, it is a challenge to recognize substandard drugs and counterfeits by applying a

single analytical method, and individual solutions to these problems are applied [34]. Only drugs having a lower content of the API than the declared one can be easily detected.

1.6 Investigated separation techniques

Two separation techniques, i.e. High-Performance Thin Layer Chromatography (HPTLC) and High-Performance Liquid Chromatography (HPLC), were applied for developing new methods for the separation and quantitative determination of sulfalene, sulfadoxine, pyrimethamine, and primaquine as well as their respective impurities and potential replacements in case of counterfeiting.

1.6.1 High Performance Thin-Layer Chromatography (HPTLC)

HPTLC is an advanced form of TLC which is commonly hyphenated with a suitable densitometer. The analysis is conducted using chromatographic plates having a smaller and narrowly distributed particle size of about 2-10 µm compared to standard TLC plates having 2-25 µm. Thus, a great number of theoretical plates is available for separation [35]. A typical HPTLC system includes a silica-coated glass plate; a chromatographic development tank; a solution applicator or spotting device; devices for controlling the relative humidity for conditioning the stationary phase; and devices for the application of reagents and heating of the plate in case the test require derivatization after development. An electronic documentation system of the densitometric analysis of the chromatogram is also provided. HPTLC may be useful in the analysis of compounds that lack a strong UV chromophore and thus require derivatization to make them detectable. This is exemplified by the quantitative determination of artemether and artesunate after derivatization with an appropriate reagent (**Figure 8**). However, it was concluded in this research work that even though the application of a derivatization agent that is quite simple, it is easily biased and might deliver inaccurate results. The Ph. Eur. provides an HPTLC monograph for the quality testing of herbal drug preparations [29], and this technique has also been used for the quantitative determination of chloroquine, primaquine, bulaquine, amodiaquine, artesunate, sulfadoxine, sulfalene, and pyrimethamine [36-40].

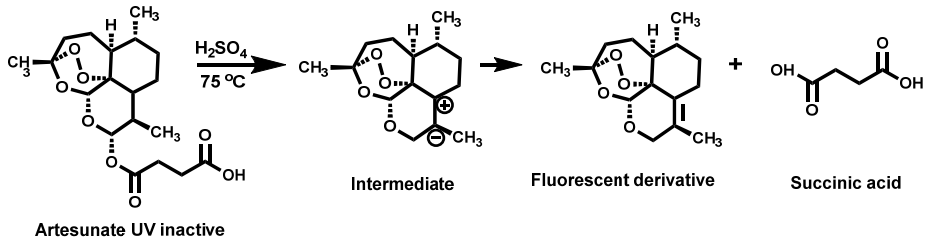

Figure 8: Reaction of artesunate with a mixture of methanol and sulphuric acid (19:1, %v/v) to form a fluorescent derivative.

1.6.2 High Performance Liquid Chromatography (HPLC)

HPLC is widely applied in pharmaceutical analysis and represents the most established separation technique [35]. In HPLC a liquid mobile phase is pumped under pressure through a stationary phase contained mostly in a stainless steel column. A typical HPLC system consists of a solvent reservoir, a pump, a sample injection device (manual or automatic), a column, a column oven, a detector, and a data capture system which mostly is a PC with software suitable for processing chromatographic data. Most commonly used detectors are ultraviolet/visible (UV/vis), or diode array spectrophotometers. Fluorescence detectors, differential refractometers (RI), electrochemical detectors (ECD), light scattering detectors, charged aerosol detectors (CAD), mass spectrometers (MS), and radioactivity detectors [29, 35] can also be applied.

Based on the polarity of the stationary and the mobile phase, there are basically two types of HPLC separations, i.e. normal and reversed phase [29, 35, 41]. Normal phase HPLC utilizes polar stationary and non-polar mobile phases, therefore non-polar compounds are eluted more rapidly, whereas in reversed-phase chromatography, a non-polar stationary and a polar mobile phase is applied. The use of normal phase HPLC is limited as most drug molecules (>90%) are polar in nature. Using reversed phase, polar compounds are eluted faster because of less affinity with the stationary phase shortening the analysis time [29, 35, 41].

Silica gel and octadecylsilane (ODS) silica gel are the commonly used column packings for normal and reversed phase, respectively. The ODS silica gel in reversed phase is chemically

modified rendering it more hydrophobic to prevent peak tailing commonly associated with basic compounds (**Figure 9**) which results in the introduction of various alkyl groups such as octyl (C_8), octadecyl (C_{18}), and phenyl (C_6H_5). After the silanization process, the overall might be subjected to a process of blocking all accessible residual silanols functions which could not be accessible during regular silanization due to steric hindrance. This is achieved through endcapping, a process which involves the treatment of such substrates with suitable agents like chlorotrimethylsilane (TMCS), hexamethyldisilazane (HMDS), or trimethylsilylane (TMS) [29, 35, 41, 42]. In this thesis standard C_{18} - either partly or fully end capped - columns were used.

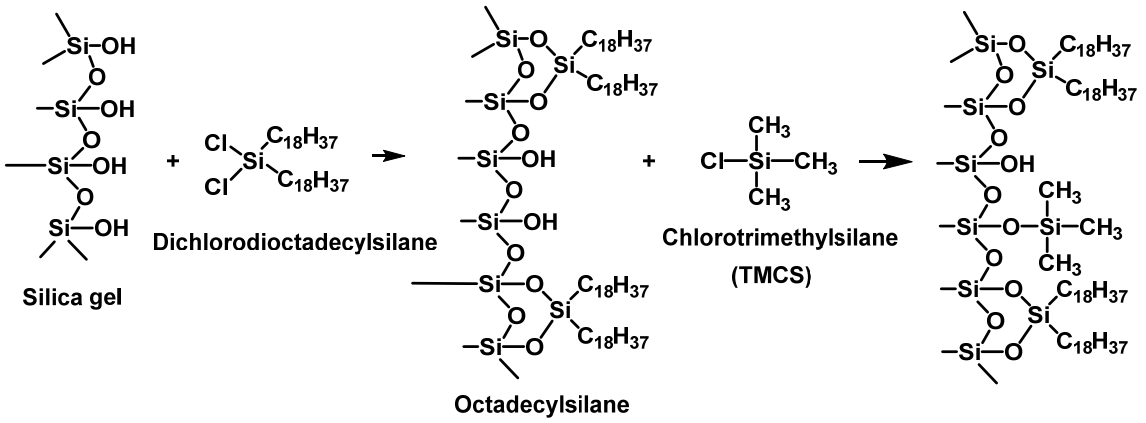

Figure 9: Silanization of free silanols in silica gel with octadecyl alkyl groups and endcapping with TMS

2. Aim of the thesis

The main objective was to develop analytical methods for antimalarial drugs which are appropriate to identify pharmaceutical products which contain a) a lower amount of the declared API, b) a different API than labeled and c) an API of low quality, i. e. containing high amounts of known or unknown impurities. Specifically the developed methods need to be performed as easy as possible and should be suitable for determining the content of the API and evaluating the impurity profile. Currently, a lot of HPLC methods are reported for antimalarial and antibiotic drugs which utilize fully automated instruments, extremely expensive columns, and often gradient elution or expensive reagents. In contrast it was

aimed to develop methods which are adapted to the often not-optimal conditions in analytical laboratories in rural Africa. These countries are characterized by limited resources to cater for routine quality control infrastructures and thus are unable to implement neither the post-marketing surveillance programs nor meeting the requirements of compendial monographs [27, 43]. Thus, the quali- and quantitative assessment methods will be designed to run on non-automated, low-level, modular instruments equipped with standard reversed phase columns and a UV detector using a simple mobile phase (i.e., aqueous buffers and/or methanol). To achieve that, HPTLC and HPLC methods were developed for sulfadoxine, sulfalene, pyrimethamine, artesunate, amodiaquine, and primaquine and were compared with regard to precision, accuracy, and robustness. This complements a commendable work carried out by Hoellein and Holzgrabe [44] in which five HPLC methods were developed for content determination and impurity profiling of five antimalarial compounds.

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3. Results

3.1 Routine Quality Control of Medicines in Developing Countries: Analytical Challenges, Regulatory Infrastructures, and the Prevalence of Counterfeit Medicines in Tanzania

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This is a review paper summarizing the current situation of medicines quality control in resource-constrained countries. Therefore people of different institutions have contributed, and I represented the Tanzania Food and Drugs Authority (TFDA).

Abstract

Counterfeit and substandard medicines still constitute a worldwide problem and do not only affect healthcare systems in low and middle income countries but also in the industrialized world. Whereas in the developed world the quality of pharmaceutical preparations is assured by a dense network of quality control laboratories utilizing modern analytical techniques the situation is completely diverse in resource constraint countries. Implementing full monograph testing according to the American or the European Pharmacopoeia represents an extreme challenge. The respective quality control organs easily become overburdened and face central problems when supplying immaculate medicines. This review collected information on the prevalence of counterfeit and substandard pharmaceuticals in Tanzania and discusses suitable analytical approaches for their analysis, e.g. non-sophisticated HPLC, low-field NMR, capillary electrophoresis, or vibrational spectroscopy. Due to the limited validity and reproducibility of field assay kits like the Minilab® the impact of precise, simple, and robust analytical methods is highlighted.

Keywords:

Counterfeit and substandard medicines; HPLC; TLC; HPTLC; Vibrational Spectroscopy; Post Marketing Surveillance; Tanzania

1. Introduction

The term “counterfeit and substandard medicines” describes a phenomenon which has been extensively studied and reported in the past. The provision of good quality medicines and pharmaceutical preparations, respectively, represents the backbone of every health care system worldwide: ensuring their availability is crucial for effective treatments and life saving therapies [1]. As the dispensed amount of e.g. an antibiotic agent is a very sensitive parameter, administering substandard medicines delays the therapeutic success, generously triggers the manifestation of resistances, and generates even more devastating burdens for the already weakened health systems [2-4]. In countries of the developing world a comprehensive quality monitoring of circulating medicines is barely possible due to relatively young health care systems, restricted laboratory capacities, weak analytical infrastructures, and chaotic distribution logistics. On the other hand, in the industrialized parts of the world the falsification of extremely expensive biotechnologically produced APIs, lifestyle medication, traditional herbal medicines and dietary supplements has become more and more relevant [5-10], indicating that the counterfeit problem is not exclusively limited to low and middle income countries any more [5, 11]. However, the majority of cases remains affiliated with those countries, e.g. in sub-Saharan Africa, where antibiotic, antiparasitic, and antiretroviral medicines are highly affected by pharmaceutical counterfeiting [11, 12]. This is very tragic because they demand for huge amounts of anti-infectives which must be available in good quality [2, 13-15]. The real extent of the problem can hardly be estimated, as a high percentage of such affairs remain undiscovered.

Evaluating the quality of active pharmaceutical ingredients (APIs) and excipients, respectively, is an integral part of contemporary pharmaceutical quality control (QC) and Good Manufacturing Practices (GMP) [16]. Usually a broad variety of modern analytical methodologies which allow the quantitative determination of the content and the purity of the

API are being applied. Separation techniques like thin layer chromatography (TLC), high performance liquid chromatography (HPLC), gas chromatography (GC), and capillary electrophoresis (CE) are fully established and have been added to almost all monographs of the major pharmacopoeias, e.g. the European Pharmacopoeia (Ph. Eur.), Japanese Pharmacopoeia and the United States Pharmacopoeia (USP) [17, 18]. Sophisticated spectroscopic methods like infrared (IR), near infrared (NIR) and Raman spectroscopy, nuclear magnetic resonance (NMR), and mass spectrometry have additionally gained a lot of attention within the last decades [19, 20]. Hyphenating the respective separation and detection techniques allows for drawing a very detailed picture of an analyte or an analyte mixture. The structure of new or unknown contaminants can be elucidated and determined even in very small quantities [21]. In modern quality control laboratories this huge inventory of analytical technologies is available anytime, a fact which allows an extensive product testing during production and during the distribution within the respective supply channels. This is only possible because the respective facilities have distinct features being obligatory to run modern instruments and which are not necessarily being found in every laboratory at anytime, e.g. air conditioning, running water and water purification systems, or electricity.

Although National Medicine Regulatory Authorities (NMRAs) have been implemented in almost all developing countries only few of them can be considered as being fully functional and operational, while others are at different levels of establishment. They suffer from a constant overburdening due to lacking resources, weak infrastructures, and the great workload which is due to the high turnover of medicines [22]. The Tanzania Food and Drugs Authority (TFDA) in Dar Es Salaam may be seen as one of the most advanced medicine regulatory authorities in sub-Saharan Africa. However, it is still a very young institution which is operating in a rather centralized manner (see **Table 1**).

Table 1: Cases of counterfeit and poor quality medicines revealed during routine inspections by the Tanzania Food and Drugs Authority (1999-2015).

Year	Trade Name	API	Dosage form	Observations
1999	Metakelfin	Sulfamethoxypyrazine, Pyrimethamine	Tablets	Confirmed to contain paracetamol
2000	Ampilin	Ampicillin trihydrate	Capsules	Confirmed to contain potato starch
2001	Quinine	Quinine hydrochloride	Injection	Confirmed to contain expired chloroquine manufactured in India
2005	Gentrisone	Betamethasone Dipropionate, Clotrimazole, Gentamicin sulfate.	Ointment	Confirmed to be a hand and body lotion only
2007	Ampishel	Ampicillin trihydrate	Capsules	Confirmed to contain potato starch
2007	Cialis	Tadalafil	Tablets	Confirmed to contain Tadalafil mixed with Sildenafil; packaging material colours were very bright
2008	Celesta mine	Betamethasone	Tablets	Red tape was used to seal both sides of the box; Blister pack has a different colour than the genuine product
2011	Elphedren	Ephedrine	Tablets	The products had similar batch numbers as the genuine product but different shelf lifes; "Batch" was written as "Match"
2011	Elphedren	Ephedrine	Tablets	
2011	Coartem	Artemether, Lumefantrine	Tablets	Confirmed that it was a relabelled expired product; the date of manufacturing was changed from 2007 to 2009 and the expiry date from 2009 to 2012
2011	Penizin-V	Phenoxymethylpenicillin	Tablets	The product resembled the registered Penizen-V having a similar batch number and manufacturer but different dates for manufacturing and expiry; the generic name was written "Pheoymethypenicillin" instead of "Phenoxymethylpenicillin", tablets had no penicillin odour and were stained and crushed
2011	Erythromycin	Erythromycin stearate	Tablets	Confirmed to contain ibuprofen instead of erythromycin; original labels were removed and replaced with labels indicating erythromycin tablets; labels were pale, and the word "tablets" was written as "tables"; strength appeared as 25 mg instead of 250 mg
2011	Laifin	Sulfamethoxazole, Pyrimethamine	Tablets	Confirmed to contain sulfamethoxazole instead of sulfametopyrazine; product label did not indicate manufacturer name and resembled Laefin Tablets manufactured in Kenya
2012	Eloquine	Quinine sulfate	Tablets	Confirmed to contain metronidazole
2012	"Praziquantel and Amodiaquine Hydrochloride Tablets"	Praziquantel, Amodiaquine hydrochloride	Tablets	The product was labelled to contain 600 mg praziquantel and 299 mg amodiaquine hydrochloride; the batch number was erased and product was labelled with the term "MSD".
2012	Sulxine&Primine	Sulfadoxine, Pyrimethamine	Tablets	The product was labelled with a registration number "TAN 07 512 JO1E MIC" which is the code of

another registered product.

Table 1 (continued)

2012	Diosulph	Sulfadoxine, Pyrimethamine	Tablets	The product (BN 051110) was labelled with the word “MSD”
2012	TTVIR-30	Nevirapine, Lamivudine, Stavudine	Tablets	The product (BN OC.01.85) was relabelled as if it was manufactured by TPI while it was manufactured by another facility
2013	Asmox	Amoxicillin	Tablets	The product was was not in the list of batches manufactured by Astra LifecarePvt. Ltd. (India); the shelf life is 3 years while the registered one (Asmox 250 mg Capsule) has a shelf life of 2 years
2013	Penizin-V	Pheoxymethylpenicillin	Tablets	The product name was written “PENIZIN-V” while the genuine one is “PENIZEN-V”, and the shelf life is 3 years while it is 1 year for the real one; the API was written as “Pheoymethylpenicillin Tablets B.P 250 mg” instead of “Phenoxymethylpenicillin Tablets B.P 250 mg”, and name of manufacturer as “ZenufaLaoratories Ltd”
2013	Halfan	Halofantrine Hydrochloride	Tablets	Confirmed to contain no API
2013	Metakelfin	Sulfamethopyrazine + pyrimethamine	Tablets	The content of the API and the physical appearance of the tablets differed from the genuine product; off white tablet colour instead of white.
2013	Chlorazen	Chloramphenicol	Capsules	The counterfeit product was found in jar packing while the genuine one is packed in blister packs of 10 x 10
2013	Strain I-2 Vaccine	Thermotolerant Newcastle Disease	Injectable Vaccine	The counterfeit products were packaged in different containers in terms of size and labelling, colour and font size used for product information.
2013	Asdoxin	Doxycycline	Capsules	Label colour, strength and words differed from the genuine product, and the written anatomical classification was different from genuine one
2014/15	No cases of counterfeit medicines are reported.			

2. Types of poor quality and adulterated/counterfeit products

Several definitions of counterfeit medicines exist, however the explanation of the World Health Organization (WHO) can be seen as the most elaborate which has gained a lot of international acceptance. Falsified medicines are described as “deliberately and fraudulently mislabelled with respect to identity and/or source, with the correct ingredients or with the wrong ingredients, without active ingredients, with insufficient active ingredients or with fake packaging" [11]. An alternative may be using the expression “spurious/falsely-labelled/falsified/counterfeit (SFFC) medicines“.

In general the following five categories have to be distinguished:

i. Copies of genuine brand medicines, often with correct amounts of the API;

ii. Products with wrong APIs; they may be of poor or of acceptable quality;

iii. Preparations containing no API at all;

iv. Medicines with too high or too low contents of the declared API;

v. Contamination with known and/or unknown impurities.

It may not always be possible to discretely assign a sample to one of these five subtypes. Non-conformance to GMP standards may result in the production of low quality products not meeting the respective quality requirements. One of the most common practices is the manufacture of placebo formulations not containing the declared API [23]. In addition, counterfeiters may not manufacture new products, but repack, relabel and resell authentic medicines after their original shelf life has expired.

3. The quality control of medicines in developing countries: challenges, limitations and analytical techniques being suitable for routine application

3.1 Challenges and limitations

Whereas in the industrialized world all sectors of the medicine market as well as the regulatory frameworks are highly controlled by internationally harmonized standards we may find a completely inverse situation in almost all poorly developed countries. Routine investigation of the quality of medicines demands for a highly connected network of qualified testing laboratories which are able to apply a broad spectrum of analytical techniques. In contrast, in developing countries adequately trained personnel may not be available at all, as well as the necessary apparatus, the required chemicals and reagents. This is either due to the unaffordability or logistic restrictions hindering the effective distribution of the respective items. It is not uncommon that spare parts have to be imported and that their delivery takes up to several months. Unpredictable power breakdowns, extremely elevated temperatures and air humidity, and shortages in the supply of consumables or chemicals constitute only few reasons why almost all modern analytical instruments cannot be run in daily routine. As a consequence the quality control of medicines is either being performed in a handful of

centrally located, prequalified testing laboratories which are heavily suffering from the resulting workload or is directly outsourced to foreign countries. Both aspects hinder continuous investigations, are responsible for unexpected additional costs and pose enormous logistical challenges.

Since only a few laboratories in developing countries are able to meet international quality and laboratory standards, in 2001 the WHO introduced a qualification programme where testing laboratories, manufacturers, and their respective products and services are being registered and audited on a regular basis by WHO inspectors.

The TFDA quality control laboratory in Dar Es Salaam, the *National Quality Control Laboratory* as well as the laboratories of the *Mission for Essential Drugs and Supplies* (MEDS),and the *National Drug Authority* (NDA) in Kenya and Uganda, respectively, constitute four facilities being so-called “WHO prequalified quality control laboratories” [24].

3.2 Analytical methods being suitable for application in developing countries

Which analytical methods are suitable and which ones are usually being applied to qualitatively and quantitatively determine the quality of essential medicines in developing countries? A concise answer to this question can hardly be given, and strongly depends on the individual situation, prerequisites, and limitations which have been described above. Developing and implementing basic testing devices which work independently from laboratory facilities and electricity has been followed since the late 1990s, and in 2001 the GPHF Minilab® was introduced as a pioneering invention within this field [25, 26].

The need for robust but effective and precise protocols has also been underlined by Martino et al. [20] who summarized the use of very simple as well as highly sophisticated analytical methods and techniques for counterfeit unravelling and concluded that liquid chromatography still represents the core technology in the field of quality analysis.

As an alternative to the major pharmacopoeias, i.e. the Ph. Eur. and the USP, the International Pharmacopoeia (Ph. Int.) has been elaborated in order to collect and harmonize analytical protocols for the determination of essential drugs which are suitable for being applied in all countries worldwide. One of its major intentions must be seen within the

provision of an appropriate regulatory frame when national legislation is missing [27]. In the majority of its monographs a combination of colorimetric reactions and TLC or HPLC assays is applied. Some methods exhibit a high grade of simplicity [28], whereas others describe sophisticated methods from the major Pharmacopoeias which require a modern instrumentation and a huge inventory of chemicals and reagents.

For the analysis of counterfeit essential medicines Nayyar *et al.* [29] as well as Hamburg [30] highlighted the development of newly field methods which have been conducted within the last five years. The following sections summarize quantitative and qualitative analytical techniques which could preferably be used in developing countries.

3.2.1 Methods for qualitative analysis

3.2.1.1 Investigation of physical and galenic properties

In some studies it was shown that severe quality deficiencies could already be discovered by examining both the primary and secondary packaging as well as the appearance of the single dosage forms[31, 32]. Apparent inconsistencies in visual properties were the reason why falsified batches of the oncologic drug Sutent® (Sutinib) were discovered in Europe in 2013 by a customer [8, 33]. Even though simple, a lot of imitations can be detected in this way. Consequently, the WHO recently published a guideline suggesting critical physical, organoleptic, and haptic parameters which should be examined: the appearance of containers, blisters, the pharmaceutical preparation itself, the label printing particulars such as registration and batch numbers, manufacturing and expiry dates, security marks like holograms, as well as shape, size colour, and odour of the drug itself [34]. Having a library of reference products may facilitate to distinguish between genuine and counterfeit products, particularly when only slightest differences can be recognized.

In this context the great variability of one and the same pharmaceutical preparation, branded or generic, has to be considered. Particularly when comparing batches which have been produced for different countries a certain product may have different specifications and appearances, e.g. of the packaging or the shape and colour of the tablets. This represents a certain limitation with regard to the significance and practicability of the WHO tests.

In contrast to haptic features galenic properties like content, uniformity, disintegration, and dissolution behaviour ultimately determine pharmacokinetic parameters like bioavailability and efficacy of nearly all solid dosage forms. Numerous investigations revealed that samples containing the correct amount of the declared substances were not able to release the respective API from the tablet matrices properly [35-39]. This is commonly due to poor manufacturing practices. The respective mostly simple tests are described in all pharmacopoeias and should always be assessed.

3.2.1.2 Colorimetric assays

Utilizing chemical reagents which react with functional groups and subsequently produce a visible change of the respective test solution is a traditional way for identifying drug compounds [18]. Prominent examples constitute the precipitation of halide ions using silver nitrate or the formation of coloured azo dyes by converting primary aromatic amines into diazonium ions and coupling them with e.g. 2-naphthol. Notably these simple principles are still described in many pharmacopoeial monographs. Wet chemistry assays do not require a complex laboratory setup and are preferably conducted in standard test tubes or on spotting plates. Such determinations are very suitable for being used in pharmacies or in field laboratories where basic laboratory inventories demand for a high grade of simplicity. Such simple colorimetric tests for the identification of APIs and counterfeit medicines, respectively, have been implemented in the already mentioned GPHF Minilab® and other detection devices [40-42]. The importance of these simple assays is undoubtedly high, particularly when *qualitative* results are of primary interest [43]. However, falsely positive results may easily be obtained due to the low specificity of the testing protocols. Molecular scaffolds being responsible for the respective positive reaction may also be present in structurally similar molecules, e.g. in APIs from the same chemical group or in certain “impurities” which have been added deliberately. Those may lead to a positive reaction already in small quantities, even when the API of interest is not present at all [44].

3.2.1.3 Spectroscopic methods

Fourier transformed infrared (FT-IR), near infrared (NIR) and Raman spectroscopy as handheld devices (cf. **Figure 1**) are promising tools in the tireless fight against counterfeit products having gained a lot of attention in low and middle income countries. They can be used during customs controls, for quick quality assessments in remote areas, and for in-process controls (IPCs) during manufacturing processes [45-50]. No chemicals are required and most of them work independently from constant power supply. The respective API may be recognized in solid and liquid formulations; according to the used excipients, samples from different manufacturers can be distinguished, and branded, generic, or falsified products can be identified [45]. Leaving the sample undamaged allows further tests, e.g. chromatographic assays determining the content. However, procurement costs are quite high. Mbinze *et al.* recently showed the limitations of applying NIR and Raman spectroscopy for the content determination of liquid quinine preparations: Due to the overall low amount of excipients in the samples (<1%) the acquired spectra did not differ significantly; the respective manufacturers could not be distinguished [47]. On the other hand, Bate *et al.* Were able to identify substandard tablets during a quality assessment of antimicrobial drugs using NIR and Raman spectroscopy [51]. This might have been achieved using established techniques, e.g. HPLC, but unveils the great potential of processing particular compounds as marker substances and subsequently detecting them by means of the respective spectroscopic methods [45, 46, 52-55]. With regard to rapidity, costs, requirement of laboratory space, electricity, and consumables NIR and Raman constitute almost ideal technologies for field use. However, quantification of the API is almost impossible.

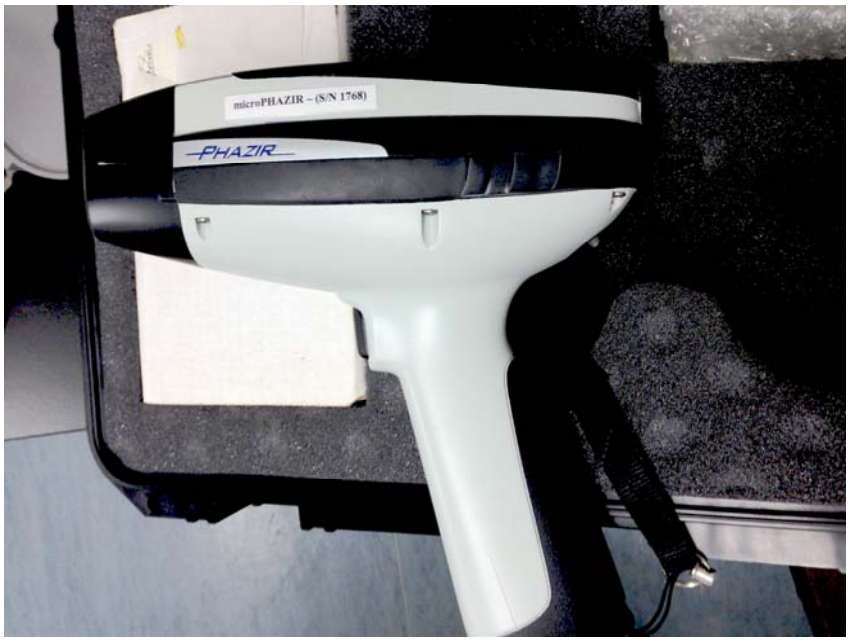

Figure 1: A handheld Raman Spectrometer (photo taken at the Muhimbili University of Health and Allied Sciences).[2]

3.2.2. Methods for semi-quantitative and quantitative analytical determinations

3.2.2.1 Thin Layer Chromatography (TLC) and High Performance Thin Layer Chromatography (HPTLC)

The role of TLC and HPTLC in pharmacopoeial testing must be regarded as almost obsolete nowadays with regard to quantification; nevertheless it is still used for the identification test and purity control of APIs in the major pharmacopoeias.

Previous issues of the Ph. Eur. described TLC assays for the determination of the related substances of many APIs, whereas the advancement HPTLC could never be fully established in the field of pharmaceutical quality control due to the difficulty of automation on a large scale. Planar chromatographic methods have been completely replaced by HPLC. Nevertheless they play an important role for the identification and semi-quantitative determination of almost all essential APIs particularly in sparsely equipped laboratories and within the framework of academic research [56]. Since the majority of the local testing institutions are facing multifactorial shortages which have been described above, TLC constitutes an ideal analytical technique in such environments. Of note, suitable TLC

[2] The photo was taken by Eliangiringa Kaale who is among the listed authors to this paper.

methods can also be found in the manuals of the GPHF Minilab® [57] where they are deployed for the semi-quantitative determination of the content of the respective APIs.

Pre-coated chromatographic plates are commercially available, and the chromatograms can usually be developed in simple glass tanks without using additional technical equipment. However, when performed manually, accuracy, reproducibility and precision of these assays strongly depend on the skills and experience of the respective analyst. Whereas qualitative assessments of APIs can be considered as unproblematic, determining the content by means of visual evaluation of the chromatograms is very difficult due to the similarity of almost all spots which are due to different concentrations (cf. **Figure 2**). This was demonstrated by investigating the quality of antimalarial compounds throughout Africa using the GPHF Minilab® protocol: only 40% of truly correct results were obtained, whereas in case of the remaining 60% falsely negative conclusions were drawn [35, 58]. In other words, the Minilab® assays could not detect the non-compliance of these samples. This was also recently shown by Fadeyi *et al.* [59].

Today, with the addition of densitometry and automatic sample application equipment along with some additional training it is possible to perform TLC and HPTLC content assessments having a repeatability and a reproducibility of the results which is comparable to those obtained with HPLC [60-62]. HPTLC protocols may provide a better sensitivity and a higher resolution of the compounds compared to classical TLC.

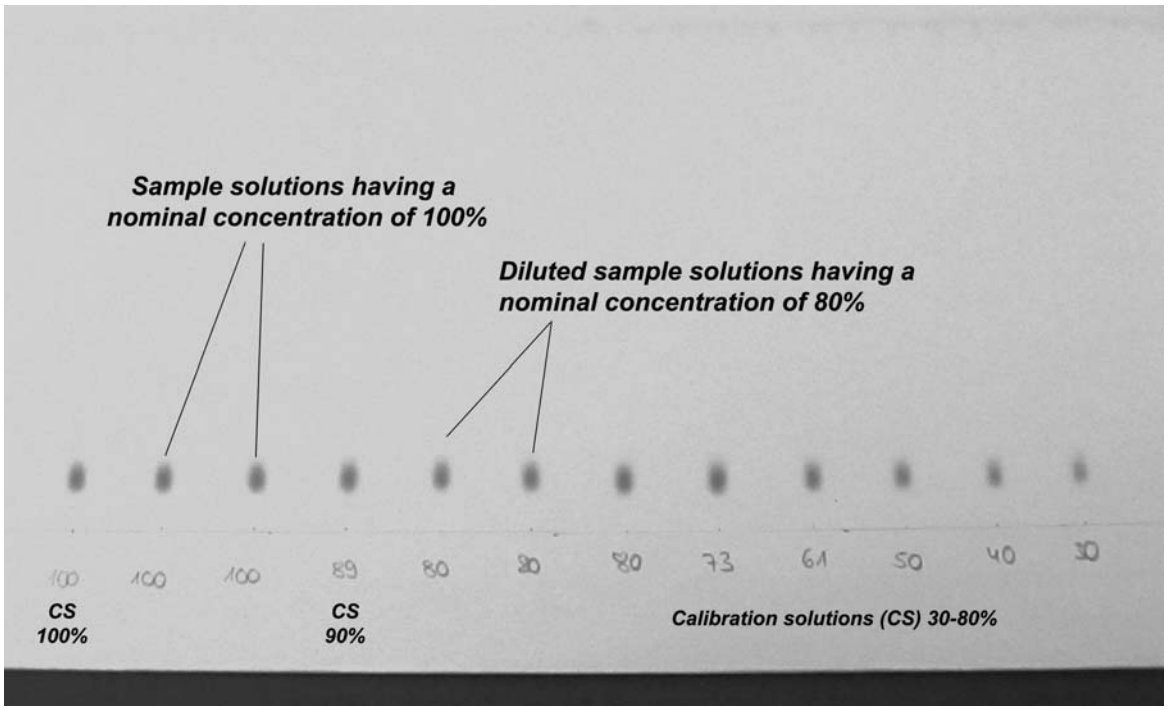

Figure 2: Thin layer chromatogram of the semiquantitative estimation of the proguanil hydrochloride content in ‘’Paludrine’’ tablets using Minilab protocol.

HPTLC can be applied for separating a huge spectrum of substances, and enables an equally sample throughput as TLC. E.g., up to 18 lanes may be applied simultaneously using a 20x10 cm plate. It can be hyphenated with other techniques and provides quantitative results which are comparable to HPLC assays [56, 60, 63-65]. HPTLC methods for the quality control of various pharmaceuticals in developing countries have been reported in the literature, including methods for the simultaneous analysis of the antituberculotic drugs isoniazid, ethambutol, rifampicin, and pyrazinamide [66], the antibiotic combination of sulfamethoxazole and trimethoprim [67], the antifungal API fluconazole [62], as well as antiretroviral drugs like lamivudine, zidovudine, nevirapine, and stavudine [61, 68]. All protocols allow the separation and quantitative determination of the respective APIs, indicating that HPTLC may be applied for the evaluation of a broad variety of pharmaceutical compounds. However, the high accuracy accounts for an expensive instrumentation like application devices, scanners, and special chromatographic plates which can be used once only, in contrast to an HPLC column.

3.2.2.2 High Performance Liquid Chromatography (HPLC)

HPLC still is the gold standard in pharmaceutical analysis exhibiting a high grade of reproducibility, sensitivity and accuracy for the qualitative and quantitative determination of all sorts of APIs [19]. Particularly in pharmaceutical sciences it has undergone a tremendous development within the last 40 years, e.g. by developing highly selective stationary phases and sophisticated instrument setups with a huge collection of extremely sensitive detectors such as mass spectrometry [69]. This emphasizes its outstanding role in solving a huge number of analytical problems in almost all scientific disciplines. Due to the fact that HPLC can be automated easily, a high sample throughput can be realized and it is highly favoured by the pharmaceutical industry [70]. Its role in developing countries has grown and it is being applied more and more: in Tanzania several major institutions have the respective modern analytical apparatus available, e.g. the TFDA headquarter and the research and development facilities of the Muhimbili University, and the *Société Générale de Surveillance* in Mwanza. The qualitative and quantitative analysis of a broad spectrum of medicines and herbal medication is conducted according to the respective compendial protocols. A countrywide implementation of this technology was not possible to date because HPLC usually demands for a complex, expensive instrumentation and a constant supply of electricity, highly purified organic solvents and reagents, air conditioned laboratory facilities and experienced analysts.

Due to all these limitations simplified conventional HPLC methods for the determination of the purity and the content might be an alternative. Therefore compendial protocols of antimalarial APIs were streamlined by avoiding expensive solvents and chemicals as well as delicate ion pairing reagents, and using easily available phosphate buffers and methanol instead [58].

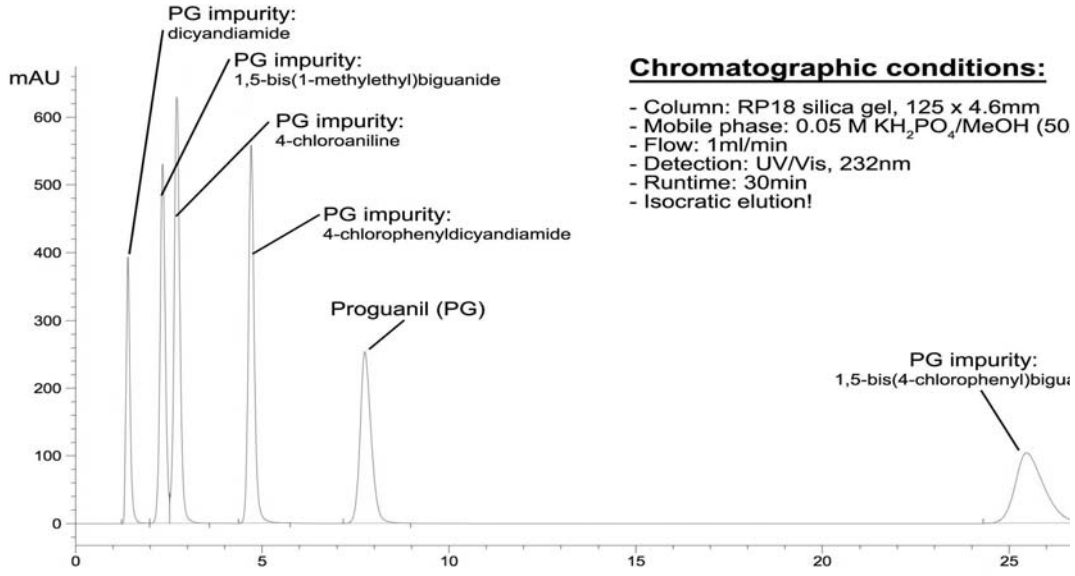

Figure 3: Separation of proguanil hydrochloride and five related compounds using a very simple, streamlined HPLC method.

Figure 3 shows the separation of proguanil hydrochloride and five related compounds using very simple chromatographic conditions; the respective method of the Ph. Eur. is more complex and requires sodium hexanesulfonate as an ion pairing reagent [71]. The validated isocratic methods can be run on robust, non-sophisticated and inexpensive HPLC instruments using short standard reversed phase silica gel columns only. They exhibit high ruggedness towards temperature and humidity shifts, composition changes of the mobile phase as well as incorrectly adjusted pH values of the respective buffer solutions. Notably, utilizing identical chemicals and columns for several APIs makes them very universal. Thus, they are an ideal intermediate testing tool which is suitable for being inserted between basic screening methods (such as the Minilab® tests) and sophisticated pharmacopoeial assays at sentinel testing centres, e.g. regional testing laboratories. Research in this area is still on-going and simplified methods for the quantitative determination of additional compounds are currently under development (Mwalwisi, Kaale, Höllein, Holzgrabe; unpublished results)[3].

[3] Currently, one paper has been published and two are still under review as reflected in the next chapters of this thesis.

3.2.2.3 Recent developments: capillary electrophoresis and low-field NMR spectroscopy

Marini *et al.* introduced a very simple low-cost capillary electrophoresis device which can be used for the quality assessment of a variety of pharmaceutical substances. They were able to develop and validate methods for the determination of quinine, furosemide, and the fixed-dose combination of trimethoprim and sulfamethoxazole. CE constitutes an interesting technology to be used in resource constraint environments, as a simple capillary is required only and a considerably low amount of solvents and buffer solutions, respectively, is being consumed. However, developing robust CE protocols may be difficult: they usually exhibit a low grade of ruggedness due to the multifactorial influences which may affect separation and migration time during analysis, e.g. capillary temperature, pH of the separation medium, or the general composition of the buffer medium [72]. Particularly when MEEKC is being applied, the properties of the running buffer may highly influence the resolution between the respective compounds [73]. In addition, the detection of an analyte or an impurity may be problematic, particularly when it is present in very small concentrations. As long as no collective of simple and robust methods is available, CE must still be considered as a domain of sophisticated laboratory testing which has not been implemented at all in Tanzania. Pages *et al.* reported the use of cryogen-free low-field NMR (LF-NMR) spectrometers which is another interesting approach for the identification of counterfeit medicines due to the quick sample preparation (only dissolution is required), the simple instrument setup, and the relatively low costs of such instruments [74] which is in the range of a medium price HPLC instrument. In addition the consumption of solvents is very low. By applying this technique Pages *et al.* were able to evaluate APIs in supposedly natural dietary supplements. Thus, LF-NMR is an interesting method for the detection of counterfeiting, especially because mixtures can be investigated (cf. **Figure 4**).

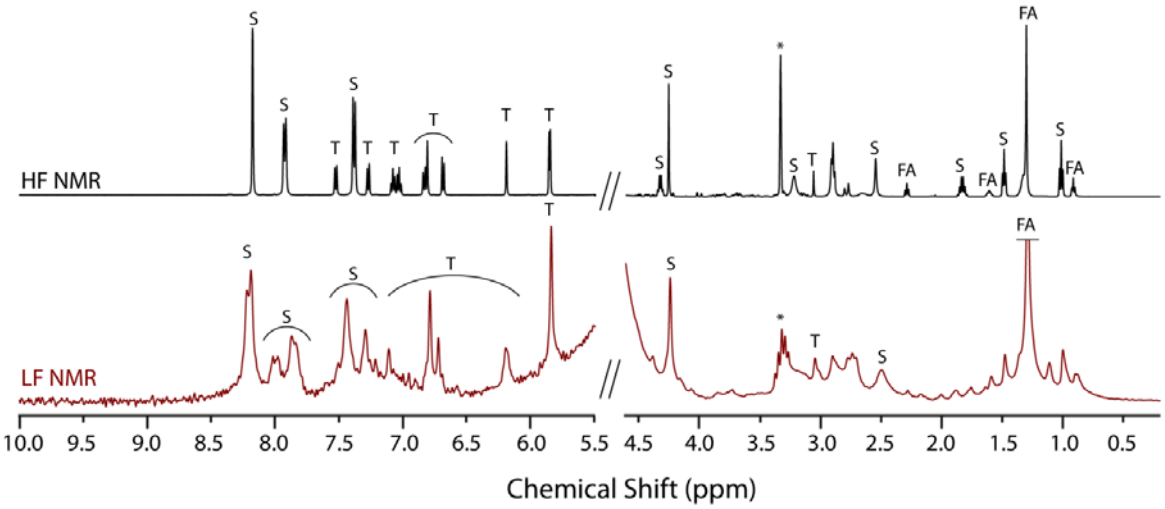

Figure 4: Comparison ^{1}H NMR spectra of the sexual enhancement dietary supplement "Indian Stud Horse" recorded in CD_2OD on the high-field (HF, 500 MHz) and low-field (LF, 60 MHz) NMR spectrometers. S=sildenafil; T=tadalafil; FA=fatty acids; *=CD_2HOD (figure kindly provided by M. Malet-Martino).

4. Medicine quality in Tanzania

4.1 Regulatory and quality control mechanisms, origin of products and medicine distribution pathways in the Tanzanian health sector

4.1.1 Regulatory and legal framework

Regulating food and medicines in Tanzania began in the colonial area in the 1930s. Three ordinances regulating the control of food, medicines, poisons and pharmacies, were the first legal rules and were superseded by the *Pharmaceuticals and Poisons Act* in 1978. Nowadays the *Pharmacy Board* regulates the quality, safety and efficacy of medicines as well as the pharmacy profession itself. Official quality control laboratories were built in the late 1990s, and the first one was opened in 2000.In 2003, the *Tanzania Food, Drugs and Cosmetic Act* was issued which constituted the establishment of the TFDA as an executive regulatory agency. Within their principle responsibilities falls controlling the production, import, distribution, and sale of food and pharmaceuticals [75]. Since its establishment a total of almost 5000 medicines and 2300 pharmacies, respectively, has been registered, and more than 20 000 applications for importations have been approved [23].

The TFDA headquarter which is located in Dar Es Salaam comprises of several directorates, reports to the secretary of the Minister of Health and is connected to a nationwide network of

so called *Zonal Offices*, resembling the European system of *Official Medicines Control Laboratories* (OMCLs) being linked to the *European Directorate for the Quality of Medicines and HealthCare* (EDQM). Although they are officially responsible of inspecting medicines, controlling import and export activities, and conducting post marketing surveillance they rather constitute administrative facilities not having the respective infrastructure for performing comprehensive laboratory analyses. Within the last years Minilabs® were donated to all of these offshore facilities in order to perform quality investigations of incoming and circulating samples. Suspicious specimens are designated to being sent to the headquarter in Dar es Salaam for full compendial analysis. However, a continuous screening may not always be guaranteed due to the problems described above. This gap again demonstrates the necessity of not only using simple testing kits, e.g. the Minilab®, but also focusing on the metamorphosis of modern "state of the art" technologies into robust field compatible methods such as the simplified HPLC [58].

4.1.1.1 Origin of medicines and distribution mechanisms within the country

Essential medicines in Tanzania are sourced from either domestic or foreign manufactures, whereby one third is being produced locally by nine manufacturers of which only two received a certification according to current GMP guidelines [76]. Thus, most of the medicines have to be imported. Particularly in remote areas controlling the import and export activities cannot be fully achieved despite being explicitly legislated by the *Guidelines for Importation and Exportation of Pharmaceutical Products and Raw Materials* [77].

The extent to which plagiarisms and poor-quality imitates are being scattered within a particular country is hardly traceable and depends on individual habits of distribution, treatment, and dispensing. Many patients initially treat themselves by purchasing the respective pharmaceutical preparations from supermarkets, kiosks, street vendors, or other unreliable sources [78] where medicines are commonly being dispensed individually from bulk packages and sold in small plastic bags under poor storage conditions (cf. **Figure 5**). The origin as well as the quality of the respective medication is hardly retraceable.

In order to close the huge supply gap between urban and remote areas the establishment of *Accredited Drugs Dispensing Outlets* [79] catering for a small inventory of essential drugs and pharmaceutical preparations was piloted.

4.2 The prevalence of counterfeit and poor-quality medicines in the Tanzanian market and post marketing surveillance activities

Countless studies have been published investigating medicine quality in almost all parts of the African continent. Massive amounts of falsified products have been discovered during national and international operations, e.g. *Intellectual Property Watch*, the *Promoting the Quality of Medicines Program* by the USP, the local press, or worldwide operations of Interpol like *Mamba*, *Storm*, or *Pangea* [80-82]. Detailed information on the quality of medicines being sold in the national market can be found in reports published by Kayumba *et al.*, Bate *et al.*, Hebron *et al.*, Kaur *et al.*, or Minzi *et al.* [4, 37, 38, 83, 84].

Figure 5: Typical dispensing of medicines in Africa using small plastic bags (own picture, taken at the Institute of Pharmacy, University of Würzburg, Germany).

All authors analysed the quality of diverse antibiotic medicines using liquid chromatography; only Bate *et al.* applied semiquantitative TLC assays. Interestingly the commonly used antimalarial fixed dose combination of sulfadoxine and pyrimethamine was subject of every investigation. Artemisinin-based antimalarials were analysed by the *ACT Consortium Drug Quality Project Team*, revealing an overall failure rate of approximately 12% (see **Table 2**) [85]. Major failure rates were observed when pharmacopoeial methods were applied for

content assessment. In almost all of the cases the disintegration and dissolution behaviour were additionally checked, and again galenic deficiencies could be discovered. Unfortunately detailed analytical data on the products is generally not accessible in detail from the majority of contemporary reports.

General reviews and summaries which have been published recently as well as a few years ago reported that up to 50% of the medicines in the developing world were counterfeit, including African and Asian countries [4, 86-88]. A failure rate of 88% could be found for Malawi [89], whereas in Gabon only 0.5% of the tested antimalarials seemed to be non-compliant using the Minilab® testing assays [90]. In 2007, Atemnkeng *et al.* reported that 37.5% of the tested artemisinin-based antimalarial preparations and up to one third of commonly applied antimalarial medicines in Kenya and Congo were out of pharmacopoeial specifications [91, 92], which is consistent with those from the Tanzanian market being also very divergent in many cases. All findings indicate that the studies are hardly comparable because they highly depend on the individual study design: Major discrepancies may come from different sampling strategies, different types of APIs investigated, or different intentional backgrounds of the respective quality assessment programmes (e.g. scientific or regulatory) [93]. A quality assessment of antimalarial drugs in Ghana and Nigeria showed that applying heterogeneous analytical techniques also led to great differences of the respective testing results [94]. In order to avoid both falsely positive and falsely negative results, all samples should be analysed by using a powerful analytical method, not only those having failed the preliminary tests such as the Minilab®. It could not gain full acceptance by the WHO as an analytical investigation tool until now because such tools [95, 96] generally exhibit a low reproducibility and the quality of the testing results is strongly biased by the end user [25, 90]. Notably there has been an improvement of the quality of medicines in the Tanzanian market which is due to the introduction of a regular post marketing surveillance program of the TFDA since 2009. As can be seen from **Table 3** the failure rates of the tested samples have declined from 50% in 2009 to 0.2% in 2015 only, and year by year the number of

evaluated samples has slightly grown reaching a maximum in the period of 2014/15 [97]. Even though this trend looks promising, it has to be confirmed in the future.

Table 2: Overview of the studies describing the quality of pharmaceuticals in the Tanzanian market.

API[#]	Principal results	Reference
AM, MD, SM/TP, QU, SD/PYR	3 MD samples failed dissolution testing after 6 months 1 TP sample showed 15% content decrease after 6 months SD/PYR: A progressive decrease in SD dissolution was observed	Kayumba*et al.*, 2004 [38]
SD/PYR, AQ, MQ, AS,AE, DHA, AE/LF	Overall 32% of the samples failed assay testing; the highest failures were observed for AQ (100%), DHA (50%), AS (31%), and SD/PYR (27%)	Bate *et al.*, 2008[4]
SD PYR	All assay tests for SD and PYR were passed 1 sample failed the disintegration testing	Hebron *et al.*, 2005 [84]
SD, SMP, PYR, SM, AQ, QU, ART	Overall 12% of the samples failed the tests; the respective API was present at any time SD/PYR: 8.6% of the samples did not meet the USP specifications for SD Tablets containing SMP and PYR were analysed for PYR only, while 19.8% of these samples did not pass USP testing for PYR AQ: 7.5% barely passed assay testing QU: 23.8% did not to meet the tolerance limits for assay set by USP ART: All formulations contained the expected amount of the API Substandard drugs were found for QU (23.8%), antifolates (13.4%), and AQ(7.5%) No association could be assumed between quality of a drug and its source	Kaur *et al.*, 2008 [37]
SD, PYR, AQ	AQ: 13% of the samples failed the dissolution testing but passed the content assays SD/PYR: 11% of the samples failed during dissolution testing and 44% failed the content assay	Minzi *et al.*, 2003[83]
ACAs	No product contained an incorrect API; 4.1% of the ART components failed content testing; 12.1% of the samples failed when ART and partner components were considered	A.C.T. Consortium Drug Quality Project, 2015 [85]

#: SD=Sulfadoxine; PYR=Pyrimethamine; AQ=Amodiaquine; MQ=Mefloquine; AE=Artemether; DHA=Dihydroarmisinin; LF=Lumefantrine; ART=Artemisinin; AM=Amoxicillin, TP=Trimethoprim; MD=Metronidazole; QU=Quinine; SM=Sulfamethoxazole; SMP=Sulfamethoxypyridazine; ACAs=Artemisinin containing antimalarials

Table 3: Post marketing surveillance results of the Tanzanian medicine market from 2009/10 - 2014/15 (source: TFDA).

Years	Type(s) of medicine[#]	Number of samples			
		Received	Tested	Pass	Fail
2009/10	AB	138	60 (43.5%)	30 (50%)	30 (50%)
	AM	143	70 (49%)	70 (100%)	0 (0%)
2010/11	AM	59	9 (15.3%)	9 (100%)	0 (0%)
2011/12	ARV	58	22 (37.9%)	20 (90.9%)	2 (9.1%)
2012/13	ARV	25	12 (48%)	12 (100%)	0 (0%)
2013/14	ARV, ATB, AM, AB	554	216 (39%)	195 (90.3%)	21 (9.7%)
2014/15	ARV, ATB, AM, AB	2526	2487 (98.5%)	2481 (99.8%)	6 (0.2%)

#: ARV=antiretroviral, ATB=antituberculotic, AM=antimalarial, AB=antibiotic

5. Conclusion

Counterfeit medicines and efforts to combat their distribution still are a highly critical issue in low and middle income countries. It is essential to have a constant quality control of pharmaceuticals [89, 98]. Since the role of simple field assay kits for quantitative quality estimations remains questionable [35], the development and commercialization of robust, sensitive and simple applicable analytical approaches especially for infrastructurally limited settings is urgently needed. The superiority of non-destructive methods, e.g. vibrational spectroscopy, could be demonstrated for certain applications; however HPLC must still be considered as the gold standard in pharmaceutical quality analysis, even when simplified methods are utilized. Strengthening the laboratory capacities and capabilities, e.g. by stipulating the availability of adequately trained personnel, should be of utmost importance. Organizing the national quality control activities in an hierarchic manner, e.g. by introducing intermediate testing laboratories in between simple field screening tests and sophisticated compendial quality testing could be a promising solution. The amount of analysed medicines could be enlarged and the time period between sampling and analysis might be reduced. (cf. **Figure 6**), ultimately resulting in an improved quality of essential medicines circulating in a particular country.

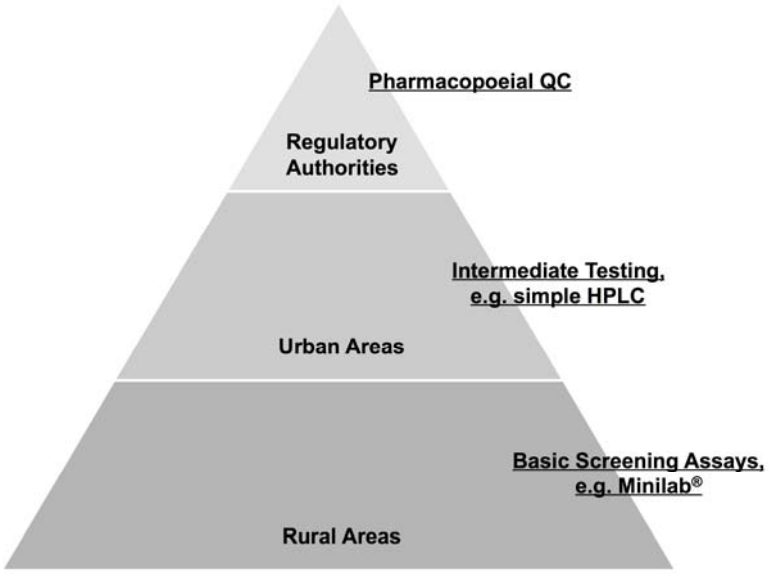

Figure 6: Proposed hierarchic testing of medicines using different analytical approaches.

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3.2 Development of a Simple, Rapid and Robust Liquid Chromatographic Method for the Simultaneous Determination of Sulfalene, Sulfadoxine and Pyrimethamine in Tablets

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Abstract

A simple, cost effective, accurate, and precise RP-HPLC method was developed for the simultaneous determination of sulfalene and sulfadoxine in fixed dose dual combinations with pyrimethamine together with their related substances. Proprietary products containing these combinations are often being prescribed in malaria endemic countries. Quantification of the active compounds and impurity profiling was achieved using two standard C_{18} columns with a mobile phase being composed of 60 % (v/v) of a 0.05M KH_2PO_4 buffer solution (pH = 2.6) and 40 % (v/v) of methanol, applying an isocratic elution mode and a detection wavelength of 215 nm. The method allows a quick quantitative determination of sulfadoxine and sulfalene and the separation of the respective impurities within a total runtime of approximately 15 min and was validated with respect to specificity, linearity, precision, accuracy, limits of detection and quantification, robustness, and stability of the standard and sample solutions. The method is simpler than the corresponding method described in the International Pharmacopoeia and the United States Pharmacopoeia in terms of being easy to apply, being less time consuming, and utilizing reagents and chemicals which are cost efficient.

Key words: Developing countries; fixed dose combination; RP-HPLC; counterfeit and substandard medicines; quality control; malaria

1. Introduction

Malaria is a parasitic disease caused by different species of *Plasmodia* which accounts for over 240 million cases and about one million deaths annually [1]. It is a major cause of morbidity and mortality particularly for pregnant women, the foetus, and the newborn. Although various highly effective active pharmaceutical ingredients (APIs) exist for the treatment of this infectious disease, the fixed dose dual combinations of sulfadoxine/pyrimethamine and sulfalene/pyrimethamine, respectively, are still commonly administered. Of note, using the combination of sulfadoxine and pyrimethamine for the intermittent preventive treatment in pregnancy is recommended by the World Health Organization (WHO) in almost all malaria endemic countries [2]. Sulfadoxine (SD), sulfalene (SL), and pyrimethamine (PYR) are well known antimalarial drugs having similar pharmacological activities (for structures see **Figure 1**). Sulfalene and sulfadoxine potentiate the effect of pyrimethamine by interfering with the tetrahydrofolate synthase and the dihydrofolate reductase in the malaria parasites [1, 3, 4], thus they are normally being sold as fixed dose combinations. Cases of poor-quality, substandard and/or counterfeit products are frequently reported in countries of the developing world [5-9]; some 50 % of the global drug market are affected by counterfeit medicines, particularly in low and middle income countries [10]. A recent quality survey performed by the WHO revealed that up to two thirds of the samples collected in sub-Saharan African countries were substandard, characterized by a lower amount of the API, failures in mass uniformity, a higher amount of related substances, or unknown impurities [11]. The use of such antimalarial drugs may result in treatment failures or delays, the development of drug resistances, and adverse effects which hinder the progress in combating malaria in these vulnerable populations [7, 8, 10]. Some studies reported the quantification of sulfadoxine and pyrimethamine applying UV/Vis spectroscopy or utilizing complexation reagents to produce coloured compounds [12-15].

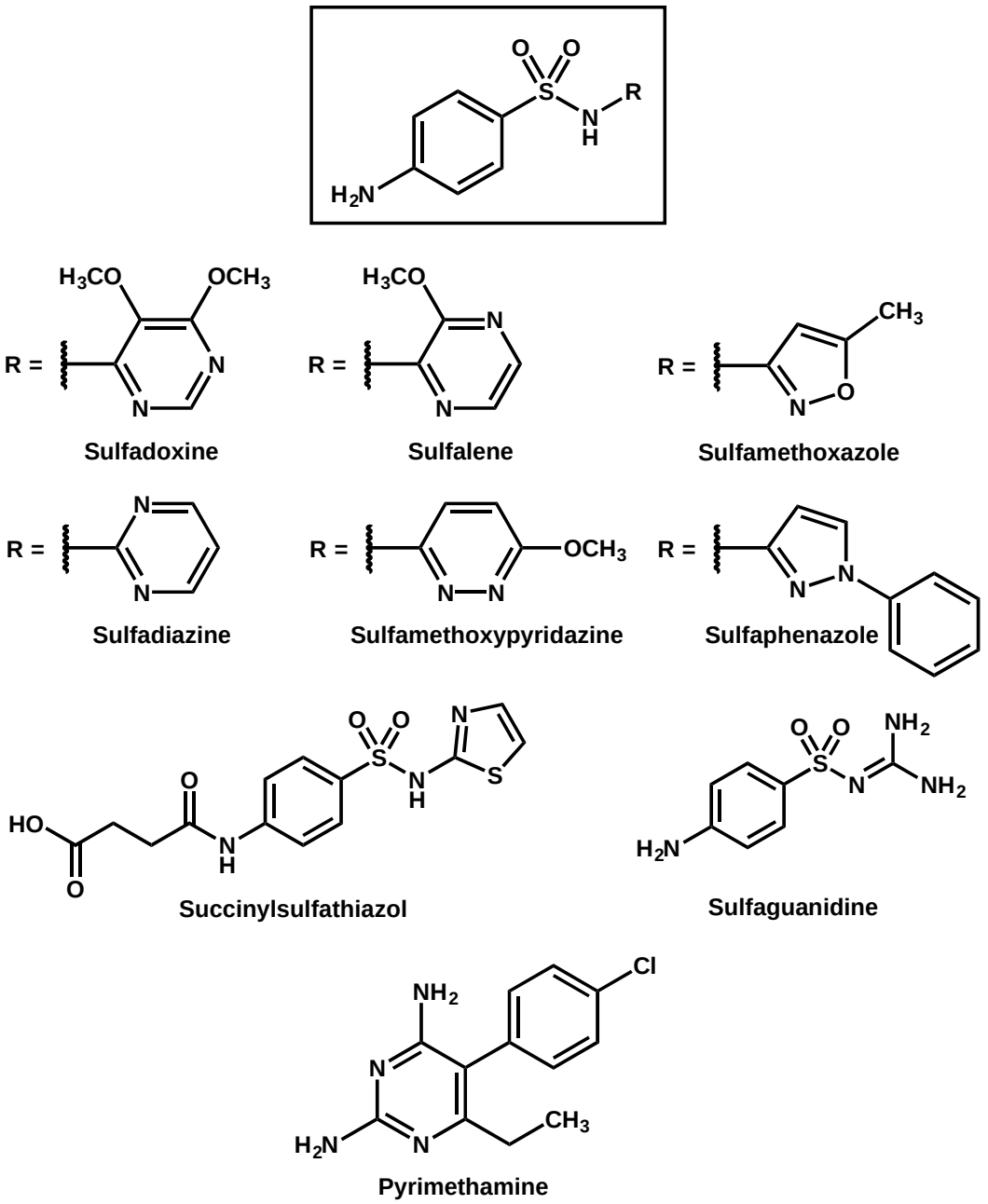

Figure 1: Chemical structures of sulfadoxine, sulfalene, and pyrimethamine as well as additional sulphonamides.

Capillary zone electrophoresis using a 100mM phosphate buffer (pH = 7.2) as background electrolyte [16], high performance thin layer chromatography on precoated silica gel plates [17], refractometric and calorimetric methods [18] as well as liquid chromatographic protocols have also been described [1, 6, 12, 19-25]. Monographs of International or the United States Pharmacopoeia are not suitable for the simultaneous quantification of the three APIs in commercially available fixed dose combinations, nor for characterizing the impurities of the respective compounds. The new method is easy to apply, less time

consuming and requires cheap chemicals and reagents only. Existing methods mostly make use of acetonitrile which is not readily available in resource constrained countries while some have been developed for biological samples only.

Having simple and robust methods available for determining the quality of essential APIs and their formulations is of particular interest for countries with limited financial resources and restricted regulatory infrastructures. The prevalence of counterfeit medicines is considerably high, and quality control laboratories often fail to apply the compendial methods, for example pharmacopoeial monographs, because the chemicals, reagents, and other consumables such as HPLC columns, acetonitrile, or triethylamine are not readily available. Therefore, suitable methods should be designed in a very streamlined and robust manner and shall utilize simple and easily available chemicals, reagents, and equipment only [26]. Of note, methods exhibiting a high grade of ruggedness can preferably be applied in developing countries, where extreme temperature fluctuations due to lacking laboratory air conditioning or very basic instrument setups are commonly encountered. We therefore aimed for the development and validation of a simple, cheap, precise, and accurate HPLC method for the determination of the three active compounds together with their related substances (cf. **Figure 2**) in commonly prescribed medicines containing either 500 mg of sulfadoxine and 25 mg of pyrimethamine, or 500 mg of sulfalene and 25 mg of pyrimethamine, respectively. The mobile phase consists of a simple phosphate buffer and methanol, while the stationary phases are two commercially available, inexpensive reversed phase C_{18} columns. In addition, it is possible to separate structurally similar sulphonamides which include sulfaguanidine, sulfanilamide, succinylsulfathiazole, sulfamethoxypyridazine, sulfamethoxazole, and sulfaphenazole from all other compounds (cf. **Figure 1**). Thus, these potential replacements can be detected in case of medicine falsifications or counterfeit products [26, 27].

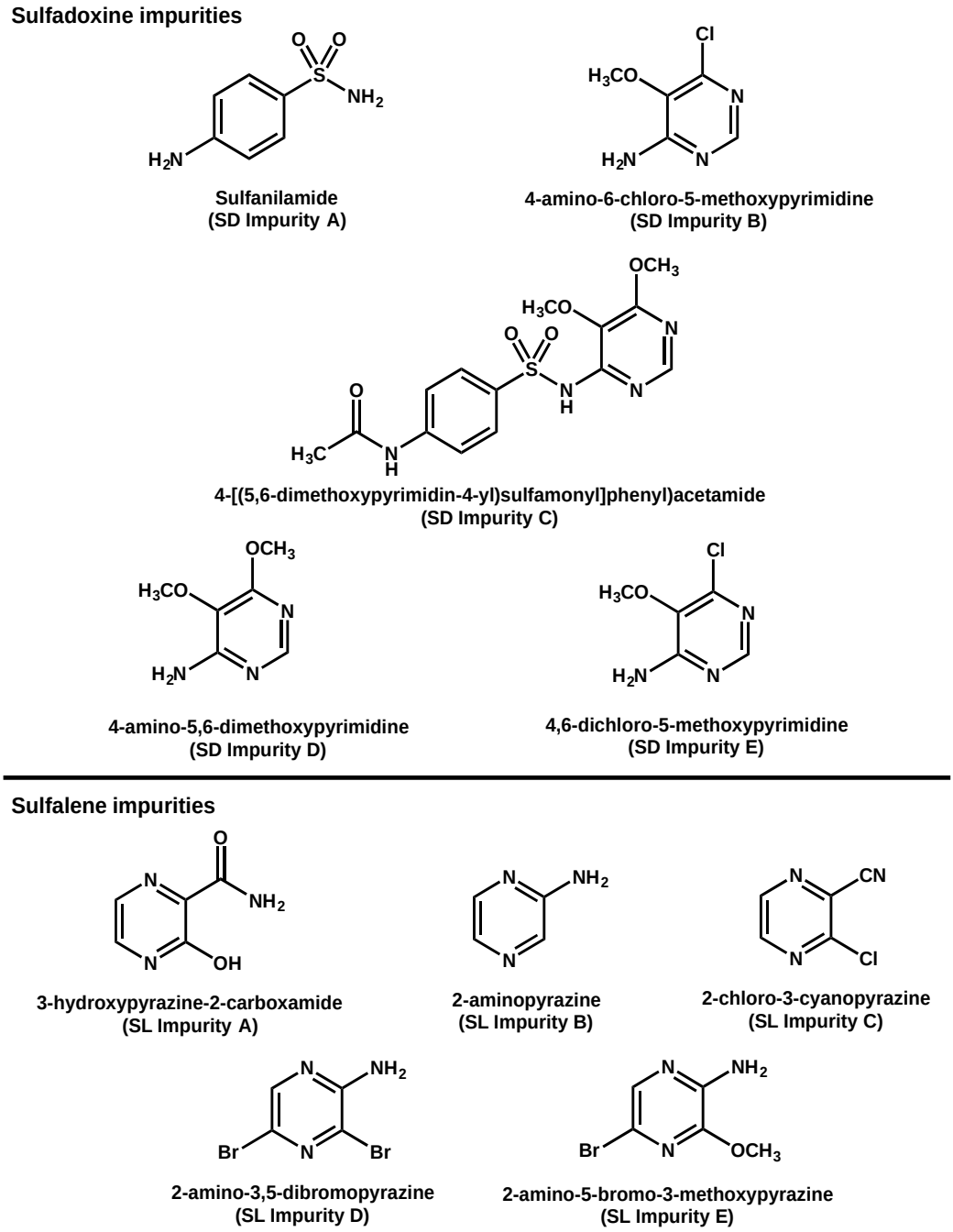

Figure 2: Chemical structures of sulfadoxine and sulfalene related substances.

2. Material and Methods

2.1 Materials

Pyrimethamine (PYR), sulfadoxine (SD), and sulfamethoxypyridazine reference standards were procured from the European Directorate for the Quality of Medicines and HealthCare (EDQM, Strasbourg, France). Sulfalene (SL) and 3-hydroxypyrazine-2-carboxamide (SL Impurity A) were procured from Tokyo Chemical Industry Deutschland GmbH (Eschborn,

Germany). Sulfaguanidine, succinylsulfathiazole, sulfamethoxypyridazine, sulfamethoxazole, sulfaphenazole, dapsone, as well as the related substances sulfanilamide (SD Impurity A), 4,6-dichloro-5-methoxypyrimidine (SD Impurity E), 2-aminopyrazine (SL Impurity B),2-chloro-3-cyanopyrazine (SL Impurity C), 2-amino-3,5-dibromopyrazine (SL Impurity D), and 2-amino-5-bromo-3-methoxypyrazine (SL Impurity E) were purchased from Sigma-Aldrich (Steinheim, Germany); 4-amino-6-chloro-5-methoxypyrimidine (SD Impurity B) was from Fluorochem ltd (Hadfield, UK), whereas 4-[(5,6-dimethoxypyrimidin-4-yl)sulfamonyl]phenyl)acetamide (SD impurity C) and 4-amino-5,6-dimethoxypyrimidine (SD impurity D) were synthesized.

Potassium dihydrogen orthophosphate (KH_2PO_4) was purchased from Carlo Erba Reagents (Rodano, Italy), methanol (HPLC grade) from Sigma-Aldrich (Steinheim, Germany), acetonitrile (HPLC grade) from Fischer Scientific UK (Loughborough, UK), glacial acetic acid (HPLC grade) from Scharlab S.L. (Sentmenat, Spain), triethylamine (Analar grade) from BDH Laboratory Supplies (Poole, England), sodium hydroxide (Analar grade) from Loba Chemie Pvt. LTD (Mumbai, India), and orthophosphoric acid (H_3PO_4) (85 %) from BDH Laboratory Supplies (Poole, England). Purified water for buffer preparation was generated by a Water Still Aquatron A4000D from Barloworld Scientific LTD (Staffordshire, United Kingdom) as well as a Milli-Q® laboratory water system from Merck Millipore (Darmstadt, Germany).

Method development and validation were carried out by employing two standard C_{18} columns with integrated precolumn (4.6mm, $C_{18,}$ 5µm particle size) (Knauer, Berlin, Germany): assay testing was performed using an Eurospher-II C_{18}H column (250x4.6mm, 5µm particle size; column A; being a polymer-bonded, high hydrophobicity and fully endcapped stationary phase) while related substances were assessed using an Eurospher-II C_{18}A column (250x4.6mm, 5µm particle size; column B; characterized by a polar endcapping and a lower hydrophobicity).

Other columns investigated included a LiChrospher 5µm RP-18 column (250 x 4.6mm, 5µm particle size; Phenomenex, Torrance, USA; column C), a Tracer Excel 120 ODSA C_{18}

column (250 x 4.6mm, 5µm particle size; Teknokroma, Barcelona, Spain; column D) and a Zorbax column (150 x 4.6mm, 3.5µm particle size; Agilent Technologies, Santa Clara, USA; column E).

The following generic versions of fixed dose combinations of sulfalene (500 mg)/pyrimethamine (25 mg) and sulfadoxine (500 mg)/pyrimethamine (25 mg) tablets were collected from the local market in Tanzania and originated from both Tanzania and Kenya: *Sulphadar* (SD/PYR; B.No.140001, Shelys Pharmaceuticals LTD, Dar es Salaam, Tanzania); *Orodar* (SD/PYR; B.No. 4A05,Elys Chemical Industries LTD, Nairobi, Kenya); *Paludar-Z* (SD/PYR; B.No.TH3003, Zenufa Laboratories LTD, Dar Es Salaam, Tanzania); *Ekelfin* (SL/PYR, B.No.3D81, Elys Chemical Industries LTD, Nairobi, Kenya); *Laefin* (SL/PYR; B.No.61206, Laboratory & Allied LTD, Nairobi, Kenya); *Malafin* (SL/PYR; B.No.130020, Shelys Pharmaceuticals LTD, Dar es Salaam, Tanzania).

2.2 Instruments

Method development and validation were conducted using two HPLC systems which were equipped with an on-line degasser, a binary pump, an automatic sample injector, and a variable UV/Vis wavelength detector (all Shimadzu, Tokyo, Japan). Robustness studies were performed on a system which additionally comprised a column oven and a photo diode array detection module; data acquisition, analysis and reporting were performed by LC solution software (all Shimadzu Tokyo, Japan). An Agilent1100 liquid chromatographic systems equipped with an on-line degasser, a binary pump, an automatic sample injector, ChemStation chromatography software for data handling, and a variable wave-length detector (Serial No. JP24019035) as well as an 1200 series liquid chromatographic system equipped with an on-line degasser, a quaternary pump, an automatic sample injector, ChemStation chromatography software for data handling, and a variable wavelength detector were employed for optimization of the method, robustness studies and impurity profiling (all Agilent, Waldbronn, Germany). Unless stated otherwise, an aliquot of 10.0 µL of the sample solutions was injected into the chromatographic system.

Other equipment included a 3510 pH meter from Barloworld Scientific LTD (Jenway, Essex, United Kingdom), an Ultrasonic bath from Wagtech International LTD (Berkshire, United Kingdom), and analytical balances from OHAUS Corporation (OHAUS, USA) and Mettler Toledo (Greifensee, Switzerland).

Mobile phases for liquid chromatography were passed through membrane filters (0.45 µm) prior to use by the aid of a vacuum pump from Wagtech International LTD (Berkshire, UK). Samples as well as standard solutions were filtered by using 0.45 µm syringe filters prior to injection into the HPLC system.

2.3 Preparation of buffer solution and mobile phases

A portion of 6.805 g of KH_2PO_4 was dissolved in 800.0 mL of purified water, and the pH was adjusted to 2.6 by adding H_3PO_4 acid (85 %). The volume was made up to 1000.0 mL with water and the pH was verified and sonicated for 15 min.

For preparation of the mobile phase for content determination 600.0 mL of the buffer solution were mixed with 400.0 mL methanol. The diluent for impurity testing was prepared by mixing 680 mL of the buffer solution with 320 mL methanol and sonicating the solution for 15 min.

2.4 Preparation of standard solutions for system suitability test (sample analysis)

10.0 mg of sulfamethoxypyridazine and 5.1 mg of pyrimethamine were accurately weighed and individually dissolved in 20.0 mL methanol. A sample of 1.0 mL of each solution was transferred into a 20.0 mL volumetric flask containing 5.1 mg of each sulfalene and sulfadoxine; after complete dissolution of the compounds the volume was made up to 20.0 mL using the mobile phase.

2.5 Preparation of sample solutions from tablets for the assay testing

20 tablets of each proprietary product were accurately weighed and ground to fine powder. An amount of powder equivalent to 62.5 mg of sulfalene or sulfadoxine (equivalent to 3.125 mg of pyrimethamine), respectively, was suspended in 30.0 mL of methanol and sonicated for 10 min. The suspensions were diluted to 50.0 mL using the same solvent and were filtered, discarding the first 10 mL. A sample of 5.0 mL of the eluate was subsequently diluted to 25.0 mL using the mobile phase.

2.6 Standard stock solutions for impurities determination

Amounts of 12.5 mg of each sulfalene and sulfadoxine impurities were accurately weighed and suspended in 10 mL of methanol, sonicated for 10 min, and the volume was made up to 50.0 mL using the diluent.

2.7 Preparation of a solution from tablet matrix for impurity profiling

20 tablets of each proprietary product were accurately weighed and ground to fine powder. An amount of powder equivalent to 250 mg of sulfalene or sulfadoxine, respectively, was suspended in 15.0 mL of methanol and sonicated for 10 min. The suspensions were diluted to the final volume (50.0 mL) using the diluent and filtered, discarding the first 10 mL. Individual volumes of the respective impurity stock solution were added to 1.0 mL of each tablet solution (cf. **Table 1**).

Table 1: Preparation of sulfadoxine and sulfalene related substances calibration and target accuracy levels.

Level	Desired conc. (mg/mL)	Amount taken from stock of solutions of set of impurities (mL)	Amount of matrix added (mL)	Final volume (mL)	Final conc.(mg/mL) (actual for each impurity based on amount weighed)
0.4 %	0.02	0.800	1.000	10	0.02
0.6 %	0.03	1.200	1.000	10	0.03
0.8 %	0.04	1.600	1.000	10	0.04
1.0 %	0.05	2.000	1.000	10	0.05
1.2 %	0.06	2.400	1.000	10	0.06
1.4 %	0.07	2.800	1.000	10	0.07

2.8 Preparation of sample solutions for impurities testing in proprietary pharmaceutical products from the Tanzania market

Solution (1): 20 tablets of each proprietary product were accurately weighed and ground to fine powder. An amount of powder equivalent to 500 mg of sulfalene or sulfadoxine, respectively, was suspended in 30.0 mL of methanol and sonicated for 10 min. The suspensions were diluted to the final volume of 50.0 mL using the diluent and filtered, discarding the first 10.0 mL

Solution (2): A sample of 0.5 mL of solution (1) was subsequently diluted to 100.0 mL using the diluent.

Solution (3): standard stock solutions were prepared as described in 2.6 above.

2.9 Method validation

Validation was conducted in accordance to the guidelines of the International Council for Harmonization (ICH) [28].

2.9.1 Validation Assay method

2.9.1.1 Linearity of the calibration line

Amounts of 50 mg of sulfadoxine and 50 mg of sulfalene were individually dissolved in 20.0 mL methanol; an amount of 12.5 mg of pyrimethamine was dissolved in 100.0 mL methanol and a portion of 10.0 mg of sulfamethoxypyridazine was dissolved in 20.0 mL methanol (stock solutions). Five calibration solutions were prepared by diluting appropriate aliquots of each stock solution to obtain concentration levels in the range of 50, 80, 100, 120, and 150% of the nominal sample concentrations. A sample of 1.0 mL of the sulfamethoxypyridazine stock solution was added to each calibration solution and the solutions were diluted to the final volumes (cf. **Table 2**).

Table 2: Preparation of sulfadoxine/sulfalene (SS), sulfamethoxypyridazine (SMX) and pyrimethamine (PYR) calibration solutions.

Calibration level	SS stock solution (mL)	PYR stock solution (mL)	SMX stock solution (mL)	Diluted to final volume (mL)	API conc. (mg/mL)	
					SS	PYR
50%	1.0	1.0	1.0	20.0	0.125	0.00625
80%	2.0	2.0	1.0	25.0	0.200	0.010
100%	2.0	2.0	1.0	20.0	0.250	0.0125
120%	3.0	3.0	1.0	25.0	0.300	0.015
150%	3.0	3.0	1.0	20.0	0.375	0.01875

2.9.1.2 Specificity

Specificity of the method was studied by preparing standard solutions containing sulfamethoxypyridazine, sulfalene, sulfadoxine, and pyrimethamine as well as sample solutions of all generic products at a nominal concentration of 0.25 mg/mL sulfalene/sulfadoxine and 0.0125 mg/mL pyrimethamine.

2.9.1.3 Recovery

Accuracy of the method was investigated by recovery studies carried out by addition of standard in which a known amount of each of the three APIs was added to the tablet sample solutions (pre-analysed by Ph. Int. monograph). These solutions were prepared by weighing powder amounts equivalent to 500.0 mg of sulfadoxine and sulfalene, respectively,

suspending them in 30.0 mL of methanol and subsequently sonicating them for 10 min. The suspensions were then diluted to the final volume of 100.0 mL using the same solvent and filtered, discarding the first 10.0 mL. A sample of 1.0 mL of each suspension was diluted to 100.0 mL using the mobile phase.

Standard stock solutions were prepared by weighing amounts of 250.0 mg of each sulfadoxine and sulfalene as well as 12.7 mg of pyrimethamine and individually dissolving them in 100.0 mL of methanol. Dilutions for the desired levels of the three APIs corresponding to 80, 100, and 120% of the nominal concentrations were prepared on three consecutive days using the mobile phase as solvent.

2.9.1.4 Precision: repeatability and intermediate precision

Six sample solutions for each *sulfadoxine/pyrimethamine* and *sulfalene/pyrimethamine* generic tablets were prepared and analysed at the nominal concentration (i.e., 0.25 mg/mL or amounts equivalent to 62.5 mg of each sulfalene and sulfadoxine, respectively). This was performed on three consecutive days while on each day all sample solutions were freshly prepared. The relative standard deviations (%RSD) were calculated for sulfadoxine, sulfalene and pyrimethamine with regard to recovery and retention times.

In order to assess intermediate precision, the procedure described above was repeated by using another analyst and different HPLC equipment. This involved measurement of freshly prepared solutions by repeating the steps above on three subsequent days and calculation of the %RSD of the data obtained.

2.9.1.5 Stability of sample and standard solutions

Stability of the standard and sample solutions was investigated by preparing all solutions at nominal concentration, i.e. 0.25 mg/mL of each sulfalene, sulfadoxine, and the corresponding amount of 0.0125 mg/mL pyrimethamine. Determination of the recovery was carried out at three different time points (0, 24, and 48 h).

2.9.1.6 Robustness

Method robustness was determined by deliberately varying the chromatographic conditions including (i) the amount of organic solvent(i.e. 30, 35, 40, and 45%), (ii) the column

temperature (i.e. 25, 30, 35, 40, and 45 °C), and (iii) the pH value of the buffer solution which was varied in the range of 2.0-3.0. In addition, columns C, D, and E which have specifications comparable to columns A and B were employed as part of this parameter.

2.9.2 Validation of the related substance method

2.9.2.1 Specificity

Specificity of the method was studied by preparing standard solutions containing sulfalene and sulfadoxine related substances as well as spiked tablet matrix solutions of the sulfadoxine/pyrimethamine and sulfalene/pyrimethamine products.

2.9.2.2 Linearity of the calibration line

12.5 mg of each sulfalene and sulfadoxine impurities were accurately weighed and dissolved in 10.0 mL of methanol and sonicated for 10 min. The solutions were diluted to a final volume of 50.0 mL using the diluent.

Six calibration solutions were prepared by diluting appropriate aliquots of each stock solution to obtain the desired concentration levels (i.e. levels of 0.4, 0.6, 0.8, 1.0, 1.2, and 1.4% of the sulfadoxine/sulfalene nominal sample solution concentration of 5.0 mg/mL; cf. **Table S3**).

2.9.2.3 Recovery

Accuracy of the method was investigated by recovery studies carried out by addition of standard in which a known amount of each of the impurities was added to the tablet sample solutions at levels of 0.4-1.4 % as described under linearity. The tablet sample solutions were prepared by weighing powder amounts equivalent to 250 mg of sulfalene or sulfadoxine, respectively, suspending them in 15.0 mL of methanol, and sonicating for 10 min. The suspensions were diluted to a final volume of 50.0 mL using the diluent and filtered, discarding the first 10.0 mL. The unspiked matrices were injected at different time intervals and used for correcting the peak areas for the spiked matrix and to ensure that interference from sample matrix is ruled out throughout the investigation.

2.9.2.4 Precision: repeatability

Six spiked sample solutions (three for each of the two levels, i.e. 0.4 and 1.0 %) were prepared for each related substance. This was investigated on three different days using individually weighed amounts of the substances as described under linearity.

2.9.2.5 Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD and LOQ were determined based on the standard deviation of the response and the slope using six concentration levels (cf. linearity). The values for LOD and LOQ were calculated as provided within the ICH guidelines [28-30].

2.9.2.6 Stability of sample and standard solutions

Stability of the standard and spiked sample solutions was investigated by preparing all solutions at a level of 0.4% of each studied substance. Determination of the recovery rate was carried out at three different time points (0, 24, and 48 h).

2.9.2.7 Robustness

Robustness was determined by deliberately varying the chromatographic conditions including the amount of the organic solvent (i.e. 30, 35, 40, 45, and 50%), the column temperature (i.e. 20, 30, and 35 °C), the pH of the aqueous component in the range of 2.4-2.8, and the flow rate (i.e. 1.4, 1.5, and 1.6 mL/min).

2.10 Quantitative determination of sulfadoxine, sulfalene, and pyrimethamine in tablets according to the International Pharmacopoeia

Mobile Phase: Volumes of 10.0 mL of glacial acetic acid and 0.5 mL of triethylamine were dissolved in 800 mL of purified water, the pH was adjusted to 4.2 by adding a solution of sodium hydroxide (40 g/100 mL), and the solution was diluted to a final volume of 1000.0 mL The pH was verified and a volume of 800 mL of this solution was mixed with 200 mL acetonitrile.

Solution 1: 20 tablets were weighed and powdered to smoothness, and an amount of powder equivalent to 125 mg of sulfadoxine was suspended in 17.5 mL acetonitrile and sonicated for 10 min. The suspension was allowed to cool to room temperature and the volume was diluted to 20.0 mL using the mobile phase. After sonification for additional 10

min, a sample of 5.0 mL of this solution as mixed with 5.0 mL of the mobile phase and passed through a 0.45 µm membrane filter, discarding the first few mL.

Solution 2: Amounts of 25.1 mg of sulfadoxine and 24.9 mg of sulfalene were dissolved in 10.0 mL of acetonitrile, the solution was sonicated until a complete dissolution was achieved and was then diluted to 25.0 mL using the mobile phase.

Solution 3: An amount of 25.1 mg of pyrimethamine was dissolved in 35.0 mL acetonitrile; the solution was sonicated and diluted to 100.0 mL with the mobile phase.

Solution 4: Samples of 10.0 mL of solution 2 and of 2.0 mL of solution 3 were mixed and diluted to 20.0 mL with the mobile phase.

The chromatographic parameters were set as described in the monograph of the Ph. Int. [19].

3. Results and Discussion

3.1 Development of a method for the quantitative determination of sulfadoxine, sulfalene, and pyrimethamine

Most methods reported in the literature make use of acetonitrile and sometimes included an organic modifier such as triethylamine, like e.g. in the monographs of the Ph. Int. [1, 6, 12, 19-25]. Such reagents may irreversibly adsorb to the stationary phase, are relatively expensive compared to simple buffer salts and may not be available in resource-limited environments. Here methanol was chosen because it is relatively inexpensive and readily available in good quality in resource-constraint countries in contrast to acetonitrile. Investigation of suitable buffer included sodium dihydrogen orthophosphate (NaH_2PO_4), potassium dihydrogen orthophosphate (KH_2PO_4), ammonium acetate, ammonium formate, glacial acetic acid and phosphoric acid and only potassium dihydrogen orthophosphate gave good results. Therefore, mixtures of phosphate buffers (20-50 mM, pH = 2-4) and methanol were tested to find an appropriate method. The influence of the column temperature on separation was also investigated between 25 to 45 ºC or 25 to 45 ºC. A different dilution solvent was used because mobile phase use resulted into blank peaks eluting close to or coeluting with some peaks of investigated substances.

Finding an optimum detection wavelength for the three APIs was crucial considering the small amounts of pyrimethamine in the tablet compared to sulfalene or sulfadoxine, respectively (1:20). The Ph. Int. makes use of λ = 227 nm [19]. Other studies reported λ = 210 nm (PYR), λ = 223 nm (SD and PYR), λ = 254 nm (SD and PYR), λ = 340 nm (SD), λ = 274 nm (SD, PYR, chloroquine, amodiaquine, and desethylamodiaquine), as well as λ = 220 nm (PYR, sulfadoxine, mefloquine, and ibuprofen) [11, 21-24, 31, 32]. The UV measurement of all compounds revealed λ = 215 nm as appropriate.

Increasing the amount of methanol in the mobile phase higher than 40% (v/v) resulted in a poor resolution between sulfamethoxypyridazine and sulfalene (R_s = 2.5 at 40% vs. 1.6 at 50%), whereas using percentages below 40% (v/v) gave an improved resolution. However, this resulted in a delayed elution of pyrimethamine (from 11.6 min at 40% to 18.7 min at 30% methanol). Although the pH of the mobile phase usually affects the separation of all analytes, the retention time of pyrimethamine is more affected than that of the sulphonamides; within the investigated pH range of 2.0 - 2.8 the shortest retention time could be found at pH = 2.6. This conclusion is supported by the chemical structure of the sulphonamides which have pKa values between 5 – 8 and thus, none will be completely ionized at the working pH of 2.6. The same applies for pyrimethamine with a pK_a value of about 7.2 and the sulfonamide nitrogen which is a weak acid having a pK_a value of 6.5, thus it will only be ionized significantly at pH > 7.5.

The following optimal chromatographic conditions were eventually found: an Eurospher-II $C_{18}H$ column (250 x 4.6 mm, 5 µm particle size; column A), being a polymer-bonded, fully end endcapped stationary phase, a mobile phase being a mixture of a 0.05 M KH_2PO_4 buffer solution (pH = 2.6) and methanol in the ratio 60:40% (v/v), ambient temperature, a flow rate of 1.5 mL/min, and a detection wavelength of λ = 215 nm, resulting in a total runtime of 15 min (cf. **Figure 3**).

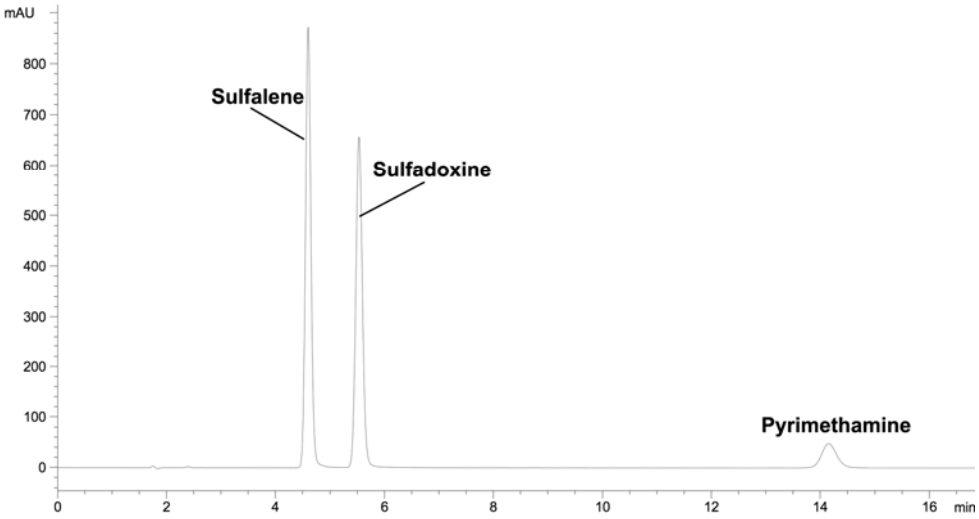

Figure 3: Chromatogram showing the separation of the three analytes using the optimized method.

The method is also capable of separating other sulfonamides, i.e. sulfaguanidine, sulfanilamide, sulfadiazine, sulfathiazole, succinylsulfathiazole, sulfamethoxypyridazine, sulfamethoxazole, and sulfaphenazole, as well as the potentially genotoxic API dapsone which might be present in the respective finished products as a fraudulent substitute [33]. However, sulfathiazole/sulfadiazine and sulfaguanidine/sulfanilamide could not be fully separated because of peak coelution (cf. **Figure 4**), which is of no relevance, because those pairs are separated from the APIs studied.

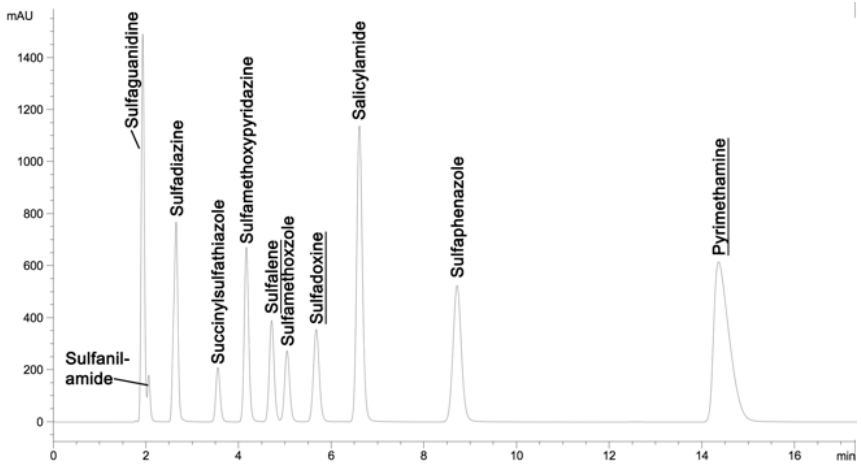

Figure 4: Chromatogram showing the separation of structurally related sulfonamides using the optimized assay method.

3.2 Validation of a method for the determination of sulfadoxine, sulfalene, and pyrimethamine

The analytical performance characteristics and validation tests, i.e. specificity, linearity, accuracy, precision (repeatability, intermediate precision), and sample solution stability, were investigated. Robustness was studied by varying the organic content of the mobile phase, the column temperature, the pH of the mobile phase, and the column brand. Where applicable, the results were compared to those obtained by applying the Ph.Int. monograph.

3.2.1 Specificity

No signal(s) in the blank injections coeluted or interfered with the three APIs. Sulfamethoxypyridazine and sulfalene as well as sulfalene and sulfadoxine were clearly separated (R_s = 2.5 and 3.3, respectively).

3.2.2 Linearity of the calibration line

The method proves to be linear within the concentration ranges of 125-375 µg/mL of sulfalene/sulfadoxine and 6.25-18.75 µg/mL of pyrimethamine, respectively. The regression data for the four analytes was as follows: Sulfalene: $y = 23628531x + 21631$; $R^2 = 1.00$; sulfadoxine: $y = 23591216x - 9159$; $R^2 = 0.9998$; pyrimethamine: $y = 51229307x - 4036$; $R^2 = 0.9999$. The statistical evaluation using a lack of fit ANOVA test indicated that the model passes the lack of fit test in the acceptable range of 0.5-0.99 (*p* value 0.999, *F* value 1.48 - 2.01 x 10^{-5}) while *F* critical was 3.885 for all three APIs.

3.2.3 Recovery and precision

The recovery for pyrimethamine in sulfadoxine/pyrimethamine combinations ranged from 99.4-100.7% compared to 99.8-100.6% in sulfalene/pyrimethamine combination products. The recovery for sulfadoxine ranged from 99.6-100.6% and from 99.8-101.1% for sulfalene, respectively, indicating an accurate method (cf. **Table S1**) [29, 34].

Table S1: Accuracy study results of the three analytes (sulfalene, sulfadoxine and pyrimethamine) determined in different tablet matrices.

Target level	Conc. added (mg/ml)	Sample conc. (mg/ml)	Total conc. (mg/ml)	Conc. Recovered (mg/ml)	% Recovery and RSD
Pyrimethamine in sulfalene/pyrimethamine tablets					
80 % (n=9)	0.01016	0.000479	0.01064	0.01070	100.6 ±0.3088; RSD=0.3 %
100 % (n=9)	0.0127	0.00060	0.01330	0.01327	99.8 ±0.4734;RSD=0.5 %
120 % (n=9)	0.01524	0.00048	0.01571	0.01574	100.2±0.3886; RSD=0.4 %
Pyrimethamine in sulfadoxine/pyrimethamine tablets					
80 % (n=9)	0.01016	0.000515	0.01068	0.01076	100.7 ± 0.7434; RSD=0.7 %
100 % (n=9)	0.0127	0.00062	0.01330	0.01330	100.0 ±0.9756; RSD=1.0 %
120 % (n=9)	0.01524	0.00051	0.01577	0.01567	99.4±0.5783; RSD=0.6 %
Sulfalene in sulfalene/pyrimethamine tablets					
80 % (n=9)	0.2000	0.009652	0.20965	0.21194	101.1 ± 0.3414; RSD=0.3 %
100 % (n=9)	0.2500	0.01196	0.26196	0.26135	99.8 ±0.4044; RSD=0.4 %
120 % (n=9)	0.3000	0.01076	0.31050	0.31156	100.3±0.3863; RSD=0.4 %
Sulfadoxine in sulfadoxine/pyrimethamine tablets					
80 % (n=9)	0.2000	0.009706	0.20971	0.21007	100.2 ± 0.6112; RSD=0.6 %
100 % (n=9)	0.2500	0.01202	0.26202	0.26102	99.6 ±1.0846; RSD=1.1 %
120 % (n=9)	0.3000	0.00978	0.30978	0.31157	100.6±0.2106; RSD=0.2 %

The intra-day precision based on the determination of recovery rates ranged from 99.8 – 100.1 % for sulfalene, from 100.2 – 100.5 % for sulfadoxine, and from 97.4 – 99.7 % for pyrimethamine. Inter-day precisions were 99.9 % for sulfalene, 100.3 % sulfadoxine, and 98.1 – 99.6 % for pyrimethamine. In case of intermediate precision, the inter-day precision was 104.9 for sulfadoxine and 101.6 % for sulfalene, while for pyrimethamine it ranged from 101.9 - 102.7 % in sulfadoxine/pyrimethamine and sulfalene/pyrimethamine products, respectively (cf. **Table 3**).

Table 3: Repeatability and intermediate precision study for sulfadoxine, sulfalene and pyrimethamine (mean values from six determinations).

	Repeatability							
	Sulphadar (SD and PYR)				Ekelfin (SS and PYR)			
Day	SD: %MR	SD: R_t (min)	PYR: %MR	PYR: R_t (min)	SL: %MR	SL: R_t (min)	PYR: %MR	PYR: R_t (min)
1	100.5	7.3	98.5	17.5	99.8	6.3	99.5	17.8
2	100.3	7.2	98.3	17.3	99.9	6.2	99.6	17.7
3	100.2	7.3	97.4	17.8	100.1	6.1	99.7	17.6
Mean	100.3	7.3	98.1	17.6	99.9	6.2	99.6	17.7
Std. Dev.	0.153	0.052	0.586	0.239	0.153	0.061	0.100	0.106
%RSD	0.15	0.72	0.6	1.4	0.15	0.98	0.1	0.60
	Intermediate precision							
	Sulphadar (SD and PYR)				Ekelfin (SS and PYR)			
1	105.0	7.5	100.7	18.0	101.6	6.3	102.6	18.0
2	104.7	7.4	101.1	17.6	101.5	6.3	102.7	18.1
3	105.0	7.4	103.8	17.6	101.6	6.3	102.8	17.8
Mean	104.9	7.4	101.9	17.7	101.6	6.3	102.7	18.0

Std. Dev.	0.173	0.031	1.686	0.224	0.058	0.000	0.100	0.153
%RSD	0.17	0.42	1.7	1.3	0.06	0.00	0.1	0.85

SD: sulfadoxine; SL: sulfalene; PYR: pyrimethamine; MR: mean recovery.

3.2.4 Stability of standard and sample solutions

The difference in content of the standard and sample solutions over time was determined in relation to the initial assay results. The acceptable limit is ± 2%, and the recovery should be between 98 and 102% of the initial concentration in order to demonstrate stability of the solutions [29, 34]. Results indicated that all contents of the standard and sample solutions were within the acceptable limit of ± 2% from initial observations with the exception of pyrimethamine at 24 h (+2.9%), indicating that the standard and sample solutions were stable for at least three days. According to the two-sample test for variance at a level of 0.05%, variances of the two data sets are not significantly different (cf. **Table S2**).

Table S2: Stability of standard and sample solutions

Substance Name	Peak area (limit ±2.0)		
	Initial	24hrs (Δ %)	48hrs (Δ %)
Standard solutions (assay)			
Sulfamethoxypyridazine	100	100.1 (-0.1)	100 (0)
SL	100	99.9 (0.1)	99.8 (0.2)
PYR	99.7	100.8 (-1.1)	99.8 (-0.1)
SD	99	99.5 (-0.5)	98.2 (0.8)
PYR	99.4	102.3 (-2.9)	99.3 (0.1)
Sample solutions (assay)			
SL	97.8	97.9 (-0.1)	98.3 (-0.5)
PYR	96.5	97.2 (-0.7)	97.1 (-0.6)
SD	97.1	97.2 (-0.1)	97.4 (-0.3)
PYR	96.2	96.7 (-0.5)	96.9 (-0.7)
Standard solutions (related substances)			
SD Imp.A	494.230	493.051 (0.2)	493.360 (0.2)
SD Imp.B	208.455	202.932 (2.6)	203.410 (2.4)
SD Imp.C	431.128	428.911 (0.5)	429.147 (0.5)
SD Imp.D	347.563	346.884 (0.2)	348.662 (-0.3)
SD Imp.E	234.244	241.311 (-3.0)	246.000 (-5.0)
SL Imp.A	292.770	293.062 (-0.1)	296.571 (-1.3)
SL Imp.B	465.219	468.954 (-0.8)	432.964 (6.9)
SL Imp.C (1.4 %)	1808.545	1231.124 (32)	1258.383 (30.4)
SL Imp.D	210.113	211.932 (-0.9)	203.471 (3.2)
SL Imp.E (1.4 %)	430.602	246.494 (43)	265.2765 (38.4)
Spiked solutions (related substances)			
SD Imp.A	92.1	92.4 (-0.3)	92.7 (-0.7)
SD Imp.B	96.1	96.3 (-0.2)	93.9 (2.3)
SD Imp.C	99.2	97.7 (1.5)	98.0 (1.2)
SD Imp.D	97.2	96.4 (0.8)	97.3 (-0.1)
SD Imp.E	119.4	116.0 (2.8)	115.1 (3.6)
SL Imp.A	92.6	92.1 (0.5)	92.5 (0.1)
SL Imp.B	87.7	88.1 (-0.5)	87.2 (0.6)
SL Imp.C (1.4 %)	100.4	84.7 (15.6)	87.4 (13)
SL Imp.D	91.8	94.4 (-2.8)	91.8 (0.0)

SL Imp.E (1.4 %)	105.8	74.1 (30)	85.2 (19.5)

3.2.5 Robustness

As expected, the retention time of all investigated compounds decreased as the percentage of methanol in the mobile phase was increased, significantly affecting the resolutions of all peaks particularly in the case of sulfadoxine and sulfalene (R_s = 1.67 compared to 3.28 at a methanol ratio of 40 %). The percentage of methanol in the mobile phase slightly affects both resolution and analysis time, thus the method cannot be considered to be fully robust.

Modifying the pH value (2.0 - 4.0) of the mobile phase revealed that the selected value 2.6 shows a short retention time of pyrimethamine ($R_{t=}$ 12 min vs. 26 min for lower and 15 – 26 min for higher pH values, respectively); the lowest resolution between sulfamethoxypyridazine and sulfalene could be observed in this case (R_s = 2.6 vs. 2.7 - 4.5 for other pH values). Since temperature changes can affect the retention times [35], the column temperature was varied in the range of 25-45 °C. However, no significant effects on the separation were observed. Since the use of methanol and buffer is associated with high back pressure, this was monitored throughout the method development, optimization and validation. The back pressure ranged from 260 – 275 bar which is relatively high but still within acceptable range regarding column dimensions. Using two additional columns with comparable specifications from different manufacturers revealed that an Eurospher-II $C_{18}H$ column (250 x 4.6 mm, 5 µm particle size; column A) shows a slightly higher resolution between closely related analytes, i.e. sulfamethoxypyridazine and sulfalene (R_s = 2.8, compared to 2.5 and 1.42 for end capped, a LiChrospher 5 µm RP-18 column (250 x 4.6 mm, 5 µm particle size) and a Tracer Excel 120 ODSA C_{18} column (250 x 4.6 mm, 5 µm particle size), respectively. In addition, analysis using the Eurospher-II column resulted in lower tailing factors for all analytes (i.e., 1.1 for column Eurospher-II compared to 1.61 and 1.42 for Lithospheric and Tracer Excel, respectively).

3.2.6 Survey of proprietary pharmaceutical products from the Tanzanian market

The content of three samples of each *sulfadoxine/pyrimethamine* and *sulfalene/pyrimethamine* product was analysed using the optimised method as well as the protocol for sulfadoxine and pyrimethamine tablets described in the Ph. Int. [19]. The assays were carried out in triplicate for the new developed method and in duplicate for the Ph. Int. monograph. The statistical analysis of the two sets of results by a two-sample test for variance at a level of 0.05 % indicated that they were not significantly different for all the products, except for *Ekelfin* in which slightly lower contents for pyrimethamine were obtained using the new method **(cf. Table 4)**.

Table 4: Comparison of assay results (%) using a compendial and the newly developed method

	Sulphonamide (SD/SL; Mean % ± SDV)		PYR (Mean % ± SDV)	
Generic Product	Ph. Int. Assay*	Simple method	Ph. Int. Assay*	Simple method
Paludar-Z (SD/PYR)	101.2 ± 0.283	103.4 ± 0.361	100 ± 0.141	102.8 ± 0.153
Sulphadar (SD/PYR)	99.4 ± 0.000	102 ± 0.321	100.6 ±1.131	101.3 ± 0.681
Orodar (SD/PYR)	101.9 ± 0.000	103.1± 0.451	102.6 ± 0.141	104.1 ± 0.404
Malafin (SL/PYR)	96.1 ± 0.000	97.2 ± 0.321	99.9 ± 0.141	99.0 ± 0.306
Laefin (SL/PYR)	95.6 ± 0.141	95.7 ± 1.480	98.6 ± 0.990	96.7 ± 1.704
Ekelfin (SL/PYR)	98.9 ± 0.071	100.7 ± 0.839	106.2 ± 0.071	102.9 ± 0.950

SD: sulfadoxine; SL: sulfalene; PYR: pyrimethamine; MR: mean recovery and SDV: Standard deviation.

This could probably be attributed to different tablet excipients and a matrix effect as this was not observed in the case of other *sulfalene/pyrimethamine* generic brands. Of note, the exact composition of excipients was unknown for all tested products as only few of them were declared.

3.3 Development of a method for related substances

For the development of an impurity profiling method the following measures were taken: (i) investigating chromatographic conditions used for the assay testing (i.e. using an Eurospher-II C_{18}H polymer bonding, fully endcapped with high hydrophobicity) column (250 x 4.6 mm, 5 µm particle size; column A; polymer bonding, fully endcapped with high hydrophobicity); (ii) application of another column with different stationary packing but identical dimensions (using an Eurospher-II C_{18}A column (250 x 4.6 mm, 5 µm particle size column B; characterized by a polar endcapping and lower hydrophobicity); (iii) variation of the methanol content of the mobile phase (i.e., 30, 32, 35, and 40%).

Methanol amounts below 40% (v/v) resulted in a longer analysis time and coelution of some substance peaks. Methanol amounts higher than 45% resulted in a poor resolution of the sulfadoxine/sulfalene impurities, while at 50% methanol the resolution of the compounds decreased. Both columns were found to be capable of separating all investigated substances. An Eurospher-II C_{18}A column (250 x 4.6 mm, 5 µm particle size; column B), offers a shorter analysis time compared to an Eurospher-II C_{18}H column (250 x 4.6 mm, 5 µm particle size; column A), particularly on the last eluting substances pyrimethamine (8.5 min cf. to 14 min),sulfalene Imp. D (10.0 min cf. to 16.7 min), sulfalene Imp. E (10.5 min cf. to 17.4 min), and sulfadoxine Imp. E (10.1 min cf. to 18.0 min; cf. **Figure 5**).

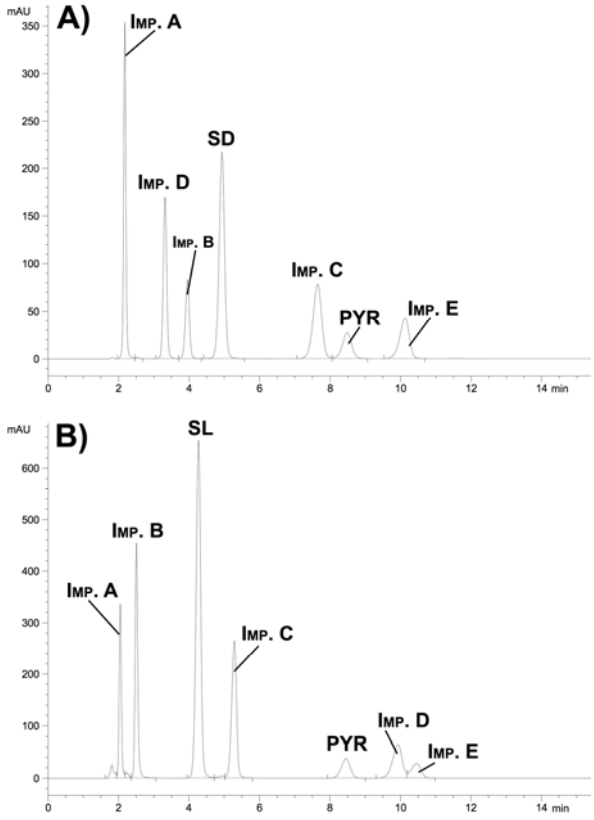

Figure 5: Chromatogram showing the separation of sulfadoxine (A) and sulfalene (B) related substances using column B and the optimized method for impurity profiling.

Furthermore, column showed a very low resolution for closely eluting substances such as sulfalene Imp. D and sulfalene Imp. E (R_s = 1.0 vs. 1.25). The Eurospher-II C_{18}A column (250 x 4.6 mm, 5 µm particle size; column B) is characterized by a polar endcapping and a

lower hydrophobicity. In contrast to the detection wavelength given in the Ph. Int. [19], the same wavelength (λ = 215 nm) was chosen as for the assay.

The following optimal chromatographic conditions were found: column B, having a polar endcapping, the mobile phase being a mixture of a 0.05 M KH_2PO_4 buffer solution (pH = 2.6) and methanol in the ratio 60:40 (v/v), ambient temperature, flow rate 1.5 mL/min, and a detection wavelength of λ = 215 nm. This result in a total runtime of 15 min.

3.4 Validation of a method for related substances

3.4.1 Specificity

No signals in the blank injections coeluted or interfered with the signals due to sulfadoxine/sulfalene and pyrimethamine or their respective impurities. All related compounds were resolved from the three APIs; the lowest R_s values were 1.84 between pyrimethamine and sulfadoxine Imp. C, and 1.04 for sulfalene Imp. D and sulfalene Imp. E.

3.4.2 Linearity of the calibration line

The method proves to be linear within the concentration ranges of 20-70 µg/mL (i.e.0.4% - 1.4% of the respective sulfadoxine/sulfalene nominal sample concentration of 5.0 mg/mL). The regression coefficients for sulfadoxine Imp. A-E and sulfalene Imp. A-E ranged from 0.988 to 0.995 and from 0.985 to 0.995, respectively (cf. **Table S3**).

Table S3: Accuracy studies results of the sulfadoxine and sulfalene related substances determined in different tablet matrices.

Target level (%)	Average added amount (mg/ml) (n=6);	recovered amount (mg/ml) (n=6);	Average added amount (mg/ml) (n=6);	recovered amount (mg/ml)(n=6);
SD Imp.A			SD Imp.B	
y=22280x (±250.474) + 74.07(±8.875); R^2=0.995 (±0.0006)			y=9602x (±204.6) + 16.523 (±3.018); R^2=0.995 (±0.002);	
0.4	0.02	0.019 (92.6 % ±0.46) (0.5 %)	0.02	0.019 (95.8 % ±1.493)(1.6 %)
0.6	0.03	0.032 (105.8 % ±1.308)(1.2 %)	0.03	0.032 (106.0 % ±0.839)(0.8 %)
0.8	0.04	0.04 (99.4 %± 0.874) (0.9 %)	0.04	0.04 (99.1 %±0.404) (0.4 %)
1	0.05	0.049 (98.0 % ±1.015) (1.0 %)	0.05	0.049 (97.4 %±1.931) (2.0 %)
1.2	0.06	0.061 (101.5 %±0.896) (0.9 %)	0.061	0.061 (100.8 %±1.604) (1.6 %)
1.4	0.07	0.069 (98.6 %±0.1) (0.1 %)	0.071	0.069 (98.2 %±1.193) (1.2 %)
SD Imp.E			SD Imp.C	
y=11682.67x (±373.007) + 18.923 (±6.270); R^2=0.988 (±0.010);			y=20409x (±505.169) + 37.173 (±7.250);R^2=0.994 (±0.000);	
0.4	0.02	0.021(102.4 %±6.326) (6.2 %)	0.02	0.019 (95.9 % ±3.517) (3.7 %)
0.6	0.031	0.035 (113.5 %±4.004) (3.5 %)	0.03	0.032 (106.2 % ±0.513) (0.5 %)
0.8	0.041	0.042 (102.8 %±2.39) (2.3 %)	0.04	0.04 (99.7 % ±1.212) (1.2 %)
1	0.051	0.051 (100.1 %±2.136) (2.1 %)	0.05	0.049 (97.6 % ±0.723) (0.7 %)

1.2	0.061	0.065 (106.1 % ±0.945) (0.9 %)	0.06	0.061(101.7 %±1.952) (1.9 %)
1.4	0.071	0.074 (103.1 % ±0.404) (0.4 %)	0.07	0.07 (100.0 % ±1.680) (1.7 %)
SD Imp.D			SL Imp.A	
y=16293.667 (±471.038) + 48.423 (±7.805); R^2=0.993 (±0.001);			y=14626x (±1170.405) + 38.67 (±2.390); R^2=0.989 (±0.009);	
0.4	0.02	0.019 (95.2 %±2.8) (2.9 %)	0.02	0.02 (95.8 % ±8.986) (9.4 %)
0.6	0.03	0.033 (107.5 %±0.666) (0.6 %)	0.03	0.032 (104.6 %±3.073) (2.9 %)
0.8	0.04	0.041 (100.8 % ±1.124) (1.1 %)	0.041	0.04 (98.5 % ±2.438) (2.5 %)
1	0.051	0.05 (98.2 %±0.794) (0.8 %)	0.051	0.048 (94.3 %±0.666) (0.7 %)
1.2	0.061	0.062 (102.0 % ±2.406) (2.4 %)	0.061	0.061 (99.3 %±1.850) (1.9 %)
1.4	0.071	0.071 (100.4 % ±1.682) (1.7 %)	0.072	0.069 (96.9 %±3.055) (3.2 %)
SL Imp.B			SL Imp.D	
y=21557x (±2320.476) + 64.917 (±23.420); R^2=0.995 (±0.003);			y=10261.67 (±831.424) + 29.7 (±8.207); R^2=0.995 (±0.002);	
0.4	0.02	0.019 (94.2 % ±5.672) (6.0 %)	0.023	0.021 (94.2 % ±2.506) (2.7 %)
0.6	0.03	0.033 (108.4 % ±1.562) (1.4 %)	0.034	0.036 (106.5 % ±0.907) (0.9 %)
0.8	0.041	0.042 (103.9 % ±0.866) (0.8 %)	0.045	0.046 (101.2 % ± 0.954) (0.9 %)
1	0.051	0.051 (100.4 % ±1.744) (1.7 %)	0.057	0.055 (97.5 % ±0.577) (0.6 %)
1.2	0.061	0.064 (105.6 % ±1.665) (1.6 %)	0.068	0.070 (103.5 % ±0.874) (0.8 %)
1.4	0.071	0.074 (103.8 % ±3.980) (3.8 %)	0.079	0.08 (101.1 % ±1.747) (1.7 %)
SL Imp.C			SL Imp.E	
y=24386x (±287.7) + 61.93 (±18.597); R^2=0.993 (±0.004);			y=5723.667x (±902.472) +14.772(±6.236);R^2=0.985 (±0.008).	
0.4	0.02	0.019 (99.4±4.153) (4.2 %)	0.02	0.021 (100.3 %±5.329) (5.3 %)
0.6	0.03	0.033 (107.8±1.952) (1.8 %)	0.031	0.034 (111.6 %±4.073) (3.6 %)
0.8	0.04	0.041 (102.2 ±0.265) (0.3 %)	0.041	0.043 (105.6 % ±3.281) (3.1 %)
1	0.051	0.05(98.9 % ±0.954) (1.0 %)	0.051	0.052 (102.4 %±3.889) (3.8 %)
1.2	0.061	0.063 (104.0 % ±0.416) (0.4 %)	0.061	0.064 (105.6 % ±6.657) (6.3 %)
1.4	0.071	0.072 (101.3 % ±0.874) (0.9 %)	0.072	0.074 (103.5 % ±4.911) (4.7 %)

3.4.3 Accuracy and precision

The intra-day percentage recoveries for all investigated impurities were within the acceptable limit of 85-115% and 0-15% RSD [29]. For sulfadoxine impurities, intra- and inter-day recoveries ranged from 92.0-119.3% and from 92.5-113.5%, whereas in case of sulfalene impurities the values were between 87.6 - 116.3% and 94.2-111.6%, respectively (cf. **Table S3**)**.** In case of sulfadoxine Imp. E and sulfalene Imp. E the values were above these limits on one day, i.e. a recovery of 95.8 – 119.3% (sulfadoxine Imp. E) and 96.2 – 116.3% (sulfalene Imp. E) was determined. However, the intra-day recoveries for the other two days were within the acceptable limit for both compounds. The inter-day recovery ranged from 92.5 – 113.5% for sulfadoxine Imp. E and from 94.2 – 111.6% sulfalene Imp. E, respectively (cf. **Table S3**)**.** The intra- and inter-day precision based on the determination of recovery rates ranged from 92.2-109.6% and 94.3-103.7%, respectively, for sulfadoxine impurities and from 87.0-114.7% and 95.3-105.3% for sulfalene related substances (cf. **Table S4**)

which were also within acceptable limits [29, 34]. Thus, the method was found to be precise and accurate.

Table S4: Repeatability study for sulfadoxine and sulfalene related substances (mean values from six determinations).

Target level (%)	Retention Time (min) (n=6)	added amount (mg/mL) (n=6)	recovered amount (mg/mL) (n=6)	Retention Time (min) (n=6)	added amount (mg/mL) (n=6)	recovered amount (mg/mL) (n=6)
SD Imp.A				SD Imp.B		
0.4	2.2±0.0 (0%)	0.02	0.019 (94.3 ±1.762) (1.9%)	4.0 ±0.06 (1.5%)	0.02	0.020(97.1 ±1.133) (1.4%)
1	2.2±0.0 (0%)	0.05	0.049 (98.4 ±0.651) (0.7%)	4±0.0 (0%)	0.05	0.049 (98.7 ±1.674) (1.7%)
SD Imp.E				SD Imp.C)		
0.4	10.2±0.060 (0.6%)	0.02	0.021 (103.7 ±5.352) (5.2%)	7.6±0.060 (0.8%)	0.02	0.019(96.0 ±3.547) (3.7%)
1	10.2±0.0 (0%)	0.051	0.051(99.6 ±3.110) (3.122%)	7.6±0.1 (1.3%)	0.05	0.049(97.7 ±0.760) (0.8%)
SD Imp.D)				SL Imp.A		
0.4	3.3±0.0 (0%)	0.02	0.019 (95.3 ±2.686) (2.8%)	2.0±0.0 (0%)	0.02	0.02(97.1 ±8.550) (8.8%)
1	3.3±0.0 (0%)	0.051	0.05(98.2 ±0.794) (0.8%)	2.0±0.0 (0%)	0.051	0.049(96.6 ±3.100) (3.2%)
SL Imp.B				SL Imp.C		
0.4	2.5±0.0 (0%)	0.02	0.02 (95.0 ±6.50) (6.8%)	5.3±0.0 (0%)	0.02	0.019(95.4 ±5.100) (5.3%)
1	2.5±0.0 (0%)	0.051	0.051 (101 ±1.910) (1.9%)	5.3±0.0 (0%)	0.051	0.05(99.0 ±0.700) (0.7%)
SL Imp.D				SL Imp.E		
0.4	10.1±0.1 (1%)	0.023	0.021 (97.1 ±5.180) (5.3%)	10.6±0.0 (0%)	0.021	0.023(97.1 ±9.252) (9.53%)
1	10.0 ±0.115 (1.2%)	0.057	0.056 (97.1 ±1.353) (1.4%)	10.6±0.071 (0.7%)	0.053	0.054(97.1 ±2.193) (2.3%)

3.4.4 Stability of standard and sample solutions

In case of the sample and standard solutions sulfadoxine Imp.A, sulfadoxine Imp.C, sulfadoxine Imp. D, and sulfalene Imp. A, the changes from initial observations were within the limit of ± 2.0% indicating the stability of the solutions for at least three days [29, 34] **(**cf. **Table S2)**. Other solutions need to be prepared freshly.

3.4.5 Robustness

As can be seen from **Figure 6** and as already discussed under method optimization, the method is not fully robust against changes of the percentage of methanol in mobile phase. In contrast, small changes of the column temperature in the range of 20 – 35 °C and the pH between 2.4 – 2.8 of the mobile phase only slightly affected the resolution, resulting in baseline separation.

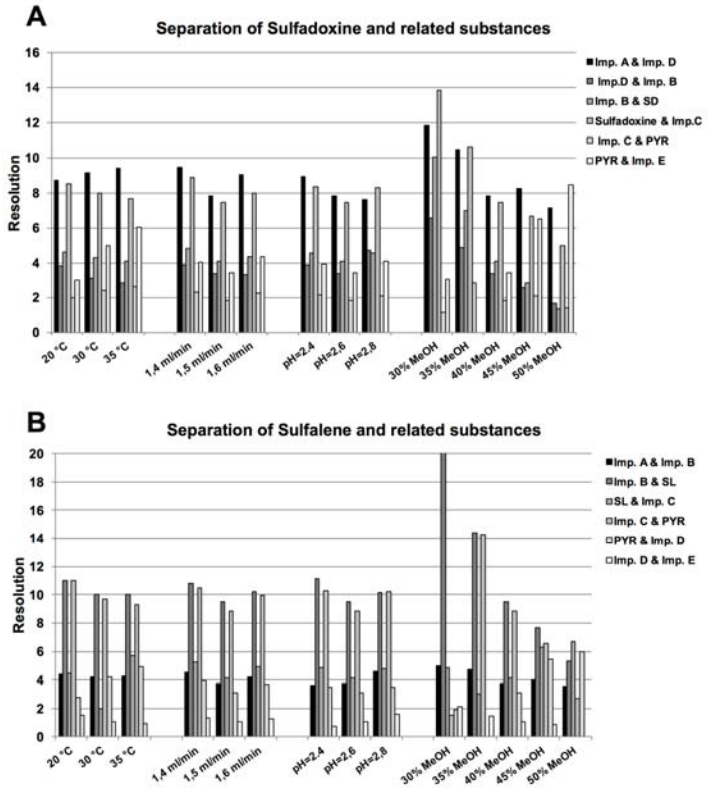

Figure 6: Robustness studies for the related substances method for sulfadoxine (A) and sulfalene (B).

3.4.6 Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD of the substances investigated ranged from 0.004-012 mg/mL, while the LOQ was 0.012 mg/mL - 0.038 mg/mL Sulfadoxine Imp. B and sulfalene Imp.B had the lowest LOD (0.004 mg/mL) and LOQ (0.012 mg/mL and 0.013 mg/mL, respectively). Sulfadoxine Imp.E showed the highest LOQ (0.038 mg/mL) compared to all studied substances (0.012-0.020 mg/mL). The percentages of impurities peak area from the main peak area ranged from 0.02 to 0.2 % for LOD and 0.02 to 0.7 % for LOQ (cf. **Table 5**).

Table 5: Limit of Detection (LOD) and Limit of Quantification (LOQ).

Related substances/impurities name	LOD(mg/mL)	% from (SD/SL)peak	LOQ(mg/mL)	% from main peak (SD/SL)
SD Impurity A	0.005	0.2	0.016	0.6
SD Impurity B	0.004	0.07	0.012	0.2
SD Impurity C	0.006	0.2	0.019	0.5
SD Impurity D	0.005	0.1	0.017	0.4
SD Impurity E	0.012	0.2	0.038	0.7
SL Impurity A	0.006	0.15	0.019	0.4
SL Impurity B	0.004	0.20	0.013	0.5
SL Impurity C	0.005	0.2	0.016	0.5
SL Impurity D	0.005	0.07	0.015	0.2
SL Impurity E	0.007	0.02	0.020	0.02

3.4.7 Related substances/synthetic impurities testing

One batch of each sulfalene/pyrimethamine and sulfadoxine/pyrimethamine proprietary product was investigated for the presence of the aforementioned related substances as well as synthetic impurities. Sulfadoxine Imp. A (0.005%) was observed in one sulfadoxine/pyrimethamine product, while sulfalene Imp. A (0.004%) and sulfalene Imp. B (0.002%) were present in one of the sulfalene/pyrimethamine samples. Unidentified impurities were observed in both sulfalene/pyrimethamine and sulfadoxine/pyrimethamine samples with contents ranging from 0.001- 0.1% which is in accordance with the Ph.Int where the limit for any individual peak is 0.5% and 1.0% for the total [1, 19, 36].

4. Conclusion

This investigation revealed that we were able to develop a simple, cost effective, accurate, and precise liquid chromatographic method for the quantitative determination of sulfalene, sulfadoxine, and pyrimethamine in fixed dose combinations along with their related substances. In contrast to the protocols of the Ph. Int. the method is characterized by a short run time of only 15 min, requires simple reagents and chemicals only, and prove to be rather robust as has been described before for other antibiotics [19, 20]. Although being slightly affected by changes of the percentage of methanol in the mobile phase, small changes of the column temperature and the pH of the mobile phase do not influence separation and quantitation during impurity testing.

Having such a simple protocol has several advantages which are (i) reducing chemical consumption, (ii) analysing more samples in comparison to time-consuming chromatographic runs, (iii) the simultaneous quantification of the three investigated APIs and their respective impurities, and (iv) recognizing other sulphonamides which might be present as substitutes in a particular sample. In fact, combining simplified methods as powerful screening tools and complex compendial monographs as valid regulatory protocols could be a very helpful tool for enabling the routine pharmaceutical quality control particularly in low- and middle income countries.

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3.3 Development of a Simple, Rapid, and Robust Isocratic Liquid Chromatographic Method for the Determination of Pyrimethamine and its Synthetic Impurities in Bulk Drugs and Pharmaceutical Formulations

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Abstract

Background: Pyrimethamine is an important antiparasitic drug in the treatment of malaria and toxoplasmosis and is often used in combination with either sulfadoxine, sulfalene, or sulfadiazine. However, the lack of a protocol for the determination of synthetic impurities and the content of pyrimethamine limit routine quality control testing of pharmaceutical formulations. The current compendial monograph as well as methods provided by the manufacturers are relatively complex and require expensive reagents and chemicals which may not be affordable for laboratories in resource-constrained countries. Therefore, a simple, cheap, precise, and accurate isocratic RP-HPLC method for the determination of pyrimethamine together with four synthetic impurities was developed as an alternative testing protocol.

Results: Pyrimethamine and its synthetic impurities can be separated within a total runtime of 30 min. The method was linear within the concentration ranges of 0.12 – 0.740, 0.104 – 0.621, 0.120 – 0.710, 2.0 – 11.8, and 1.01 – 5.80 µg/mL for pyrimethamine, impurity A, impurity B, impurity C, and impurity D, respectively. The coefficients of determinations (R^2) for the five analytes were greater than 0.994 for pyrimethamine and all impurities. Recovery studies were in the range 89.1 – 105.1% for all substances. All tested genuine batches of pyrimethamine had impurities within the specified limits which is concurrent with results obtained from using the present manufacturer's method.

Conclusion: The newly developed method can be applied as an alternative to current compendial monographs of pyrimethamine as well as the current manufacturer method for content determination and impurity profiling of pyrimethamine in bulk drugs or proprietary formulations.

Keywords: pyrimethamine**,** RP-HPLC; quality control; bulk drug and tablet analysis; malaria; impurity profiling

1. Background

Pyrimethamine (5-(4-chlorophenyl)-6-ethyl-2,4-pyrimidinediamine), belongs to a group of antimalarial drugs that are effective against hepatic and erythrocytic forms of plasmodia by inhibiting the parasitic enzyme dihydrofolate reductase. It is used in synergistic combination with a sulphonamide, e.g. sulfadoxine or sulfalene; however, resistances have been developing within the last years. Moreover, the fixed dose combination of these compounds plays a very important role in the prevention of malaria in pregnant women. Pyrimethamine can also be combined with sulfadiazine or trisulfapyrimidine for the treatment of toxoplasmosis [1].

The current compendial monographs for pyrimethamine in the European (Ph. Eur.) and the British Pharmacopoeia (B. P.) describe the determination of the related substances utilizing thin layer chromatography (TLC). The United States Pharmacopoeia (USP) as well as the International Pharmacopoeia (Ph. Int.) do not have provisions for related substances but describe assay testing by liquid chromatography or a non-aqueous titration, respectively [2-4]. Additionally, the USP and B. P describe monographs for content determination of pyrimethamine in tablet formulations by using a UV/Vis spectroscopic method, λ = 272 and 273 nm, respectively [3, 4]. Other studies reported the determination of pyrimethamine in finished pharmaceutical products in combination with other substances such as sulfadoxine by HPLC [2, 3, 5-7], capillary zone electrophoresis [8], and high-performancethin layer chromatography (HPTLC) using precoated silica gel plates [9]. The lack of a protocol for impurities determination warranted the development and validation of a simple, cheap, precise, and accurate HPLC method for the determination of pyrimethamine together with its synthetic impurities (cf. **Figure 1**). Since this method is intended to be applied in resource-constrained countries preferably, it should require utilizing easily available and inexpensive chemicals, reagents, and basic equipment only [10-12]. Hence, the mobile phase consists of

a simple phosphate buffer and methanol, while the stationary phase is a commercially available, inexpensive reversed phase C_{18} column.

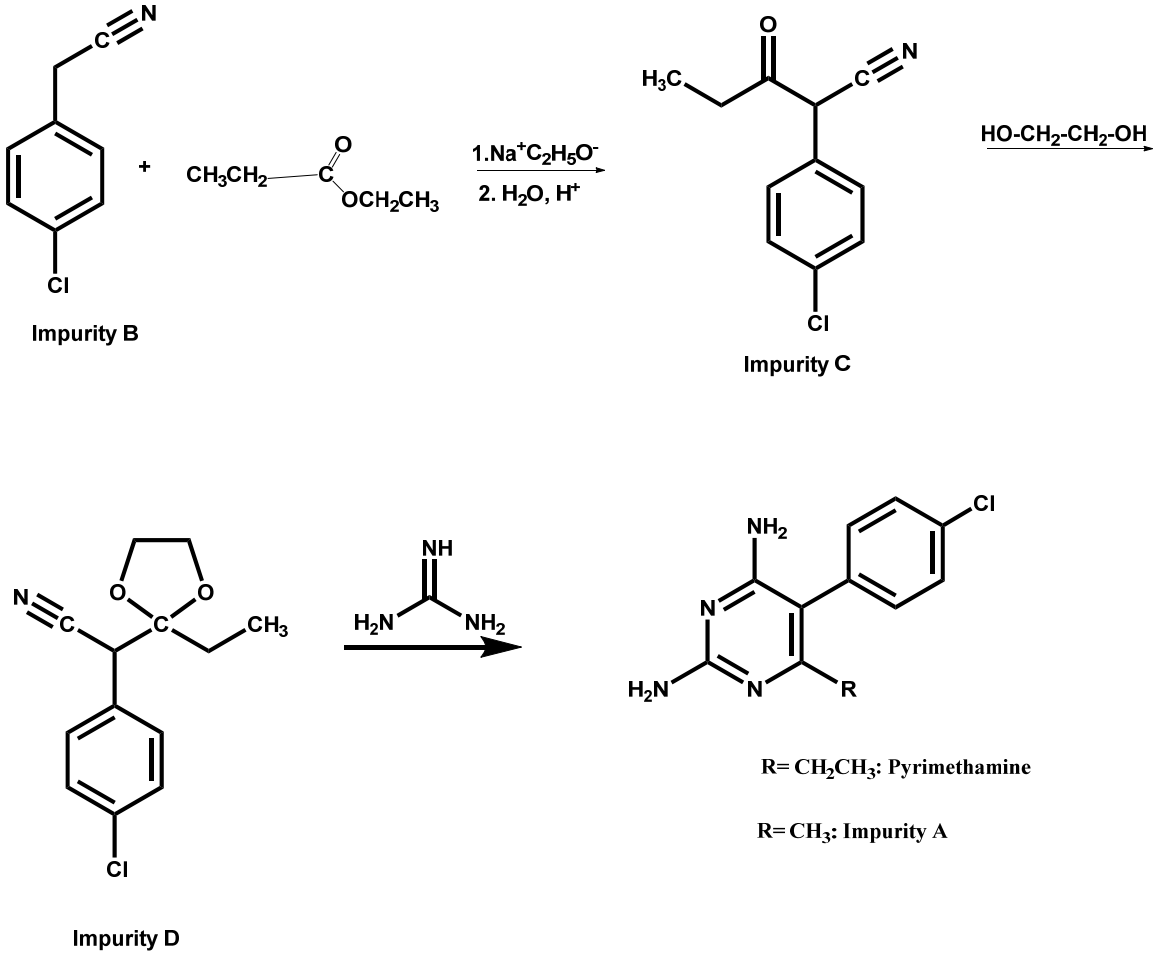


Figure 1: Pyrimethamine synthesis pathway [1, 18, 19].

2. Methods

2.1 Chemicals

Pyrimethamine, *p*-chlorobenzyl cyanide (impurity B), and α-propionyl-4-chlorobenzyl cyanide (impurity C) were purchased from Sigma-Aldrich (Steinheim, Germany), while 5-(4-chlorophenyl)-6-methylpyrimidine-2,4-diamine (impurity A) and 1-(4-chlorophenyl)-1-cyano-2-ketolbutane (impurity D), as well as pyrimethamine samples (Batch No. 1, 2, and 3), were obtained from the European Directorate for the Quality of Medicines and HealthCare (EDQM, Strasbourg, France). Potassium dihydrogen orthophosphate (KH_2PO_4) was purchased from Carlo Erba Reagents (Rodano, Italy), methanol, glacial acetic acid (HPLC

grade), and orthophosphoric acid (H_3PO_4) (85 %) were from Sigma-Aldrich (Steinheim, Germany). Purified water for buffer preparation was generated by a Milli-Q® laboratory water system from Merck Millipore (Darmstadt, Germany). Method development and validation were carried out by employing standard C_{18} columns with an integrated precolumn (each 250 x 4.6 mm, particle size 5 µm) (Knauer, Berlin, Germany): an Eurospher-II C_{18}H column (column A, being a polymer-bonded, high hydrophobicity and fully end capped stationary phase), an Eurospher-II C_{18}A column (column B; characterized by a polar endcapping and a lower hydrophobicity), as well as an Eurospher-II C_{18}P column (column C; being a polymer bonded, fully end capped silica gel, with high hydrophobicity).

2.2 Instruments

Method development and validation were conducted using an Agilent 1100 liquid chromatographic system equipped with an on-line degasser, a binary pump, an automatic sample injector, and a variable wavelength detector. A 1200 series liquid chromatographic system equipped with an on-line degasser, a quaternary pump, an automatic sample injector, and a variable wavelength detector was employed for optimization of the method, robustness studies, and impurity profiling. Data was acquired and processed using the Agilent ChemStation for LC 3D Systems (Rev. B. 03.02) [341]. For determining an unknown impurity peak, an Agilent 1000 series liquid chromatographic system as well as an Agilent 1100 series LC/MSD ion Trap software (Version 5.3) and data analysis for LC/MSD ion Trap (version 3.3) [Build 146] were used (all Agilent, Waldbronn, Germany). Unless stated otherwise, a sample of 20.0 µL of each solution was injected into the chromatographic system. Other equipment included a Metrohm AG pH meter from Deutsche Metrohm Prozessalytik GmbH (Filderstadt, Germany), an ultrasonic bath from Bandelin Electronic GmbH (Berlin, Germany), and analytical balances from Sartorius AG (Göttingen, Germany) as well as Mettler Toledo (Greifensee, Switzerland). Mobile phases for liquid chromatography, samples, and standard solutions were passed through membrane filters (0.45 µm) prior to injection into the HPLC system.

2.3 Solutions

2.3.1 Preparation of buffer solution and mobile phases

A portion of 6.805 g of KH_2PO_4 was dissolved in 800 mL of purified water, and the pH was adjusted to 2.6 by adding phosphoric acid (85%). The volume was made up to 1000.0 mL with water, the pH was verified, and the solution was sonicated for 15 min. The mobile phase was prepared by mixing 400 mL of the buffer solution with 600.0 mL methanol and sonicating the solution for 15 min.

2.3.2 Preparation of standard stock solutions

Samples of 2.50 mg of Pyrimethamine, 2.50 mg of impurity A, 2.50 mg of impurity B, 2.50 mg of impurity C, and 2.50 mg of impurity D were individually dissolved in 25.0 mL of the mobile phase, sonicated for 15 min, and then diluted to 50.0 mL using the same solvent (50 ppm). A sample of 1.0 mL of each impurity A, impurity B, as well as the Pyrimethamine stock solution was individually diluted to 20.0 mL resulting in a concentration of 2.5 ppm.

2.3.3 Preparation of sample solutions for the impurity testing

A sample of 5.0 mg of each batch of Pyrimethamine was dissolved in 5.0 mL of the mobile phase and sonicated for 15 min. The volume was made up to 10.0 mL using the same solvent and giving a concentration of 0.50 mg/mL.

2.3.4 Standard solution for impurities determination

Samples of 2.50 mg of pyrimethamine, 2.50 mg of impurity A, 2.50 mg of impurity B, 2.50 mg of impurity C, and 2.50 mg of impurity D were individually dissolved in 25.0 mL of the mobile phase, sonicated for 15 min, and then diluted to 50.0 mL using the same solvent (50 ppm). Samples of 1.0 mL of the standard stock solutions were diluted to 100.0 mL with the mobile phase resulting in a concentration of 0.5 mg/mL.

2.4 Method validation

Validation was conducted in accordance with the guidelines of the International Council for Harmonization (ICH) [13].

2.4.1 Specificity

Specificity of the method was studied by preparing standard solutions containing impurity A, impurity B, impurity C, impurity D, pyrimethamine, as well as sample solutions of the pyrimethamine batches at working concentrations of 0.0005 mg/mL and 0.500 mg/mL, respectively. The specificity of the method was assessed by comparing the chromatograms and retention times obtained using standard solutions against that of sample solutions to ensure that none of them coeluted with the API or with each other.

2.4.2 Linearity of the calibration line

Six calibration solutions were prepared by diluting appropriate aliquots of each stock solution described (cf.1.4) above to obtain the desired concentration levels (i.e. levels of 0.02 to 2.4% of the pyrimethamine solution having a nominal concentration of 0.5 mg/mL; cf. **Table 1**).

2.4.3 Recovery

The accuracy of the method was investigated by recovery studies carried out by the addition of a standard in which a known amount of each of both active pharmaceutical ingredient (API) and the investigated impurities were added to the mobile phase at levels of 0.02 – 2.4% of pyrimethamine nominal concentration (0.500 mg/mL).

2.4.4 Precision: repeatability

Six independent solutions were prepared at the Limit of Quantification (LOQ) and two other levels of the calibration concentrations for each impurity (i.e. 0.03, 0.10, and 0.15% for pyrimethamine; 0.02, 0.08, and 0.12% for impurity A; 0.24, 0.10, and 0.14% for impurity B; 0.4, 1.6, and 2.4% for impurity C; and 0.2, 0.8, and 1.2% for impurity D). This was investigated on three consecutive days using separately weighed amounts of the impurity.

2.4.5 Limit of Detection (LOD) and Limit of Quantification (LOQ)

Limit of Detection (LOD) and Limit of Quantification (LOQ) were determined based on the standard deviation of the response and the slope using six concentration levels (cf. linearity). The values for LOD and LOQ were calculated as provided within the ICH guideline “Impurities in New Drug Products” [13].

2.4.6 Stability of sample and standard solutions

Stability of the standard and sample solutions was investigated by preparing all solutions at the working concentration levels 0.104, 0.119, 1.960, 2.022, and 0.765 µg/mL for impurity A, impurity B, impurity C, impurity D, and pyrimethamine, respectively. Measurements were done in duplicate and determination of a possible decrease of the initial peak area was carried out at three different time points (0, 24, and 48 h).

2.4.7 Robustness

Robustness was determined by slightly varying the chromatographic conditions including the amount of the organic solvent at ± 5% (i.e., 57, 63, and 66%), the column temperature (i.e., 20, 25, 30, 35, 40, and 45 °C), the pH of the aqueous component at ± 0.2 pH units (i.e. 2.4, 2.8, and 3.0), and the flow rate was adjusted by ± 0.1 mL/min (i.e., 1.1, 1.2, and 1.3 mL/min).

2.5 Quantitative determination of Pyrimethamine synthetic impurities according to the manufacturer method

Stationary phase: a Eurospher-II $C_{18}H$ column (0.25 m x 4.6 mm, 5 µm particle size). Mobile phase A: 2.72 g of potassium dihydrogen orthophosphate was dissolved in 1000.0 mL of distilled water and adjusted to pH 8.0 using ammonia. Mobile phase B: acetonitrile. The diluent was prepared by mixing 500.0 mL of mobile phase A and 500.0 mL of acetonitrile and sonicating the solution for 10 min.

Test solution: 10.0 mg of each batch of pyrimethamine was dissolved in 4 mL of the diluent, sonicated for 10 min and diluted to 10.0 mL with the same solvent. Reference solution (a): 10.0 mg of pyrimethamine, 10.0 mg of impurity A, 10.0 mg of impurity B, and 10.0 mg of impurity C were dissolved in 50.0 mL of the diluent, sonicated and diluted to 100.0 mL with the same solvent. Reference solution (b): 10.0 mg of impurity D and 10.0 mg of toluene were dissolved in 50.0 mL of the diluent, sonicated for 10 min, and diluted to 100.0 mL with the same solvent. Reference solution (c): 10.0 mL of solution (a) and 5.0 mL of solution (b) were diluted to 100.0 mL with solvent mixture. Reference solution (d): 1.0 mL of the reference solution (c) was diluted to 10.0 mL with the solvent mixture.

A gradient programme was employed: 0 – 6 min: 65% A; 6 – 20 min: 65 → 40% A; 20 – 30 min: 55% A; 30 – 35 min: 55 → 65% A; 40 min: 65% A. Analysis was carried out at 30 °C applying a flow rate of 1.0 mL/min, and a detection wavelength of λ = 210 nm. The injection volume was 10 µL.

2.5 Investigation of an unknown Pyrimethamine synthetic impurity

The structure of the unknown impurity (eluting before impurity A with method (a) and after with variation of method (b)) was elucidated by applying an LC/MS (ion trap) measurement utilizing a 150 mm Eurospher-II C_{18}A column (5 µm particle size, internal diameter4.6 mm) in isocratic mode with a flow rate of 1.0 mL/min. The mobile phase contained 55% (v/v) of 0.05 M ammonium formate (pH = 2.7) and 45% (v/v) of methanol; UV detection was carried out at λ = 254 nm. The mass spectrometer was operated in positive ion mode and the capillary temperature was set at 325 ºC. The nebulizer gas pressure was set at 15 psi and the dry gas flow was set at 5.0 mL/min. The full scan began at 100 to 2200 m/z, with averages of 7 spectra.

3. Results and Discussion

The aim of the study was to separate four impurities of Pyrimethamine being starting materials (impurity B) and intermediate products (impurity A, C and D) of synthesis as well as toluene (solvent) from the main peak. Impurity A is observed when ethyl propionate is contaminated with ethyl acetate (**Figure 1**).

3.1 Development of a method for the quantitative determination of Pyrimethamine and its synthetic impurities

The previously published method for the simultaneous analysis of the content of sulfalene, sulfadoxine, and pyrimethamine was used as a starting point for the separation of pyrimethamine and its synthetic impurities [14]. However, the conditions resulted in coelution of impurity C and impurity D. The optimal chromatographic conditions were eventually found by employing a Eurospher-II C_{18}H column (250 x 4.6 mm, 5 µm particle size; column A), being a polymer-bonded, high hydrophobicity and fully end capped stationary phase, and a mobile phase being a mixture of a 0.05 M KH_2PO_4 buffer solution (pH = 2.6) and methanol in

the ratio 40:60% (v/v).The analysis was carried out at 30 ºC, applying a flow rate of 1.2 mL/min, and a detection wavelength of λ = 215 nm, resulting in a total runtime of 30 min. Representative chromatograms which have been recorded using the newly developed as well as the current method provided by the manufacturer are shown in **Figure 2** and **3**. In order to develop a method which might also be used with LCMS, other buffers compatible with mass spectrometry such as ammonium acetate, glacial acetic acid, phosphoric acid, sodium acetate, and ammonium formate proved to be useful for separating all other impurities except for impurity B. However, using those buffers at λ = 215 nm wavelength resulted in a high background noise.

The influence of the amount of organic solvent, i.e. methanol, the column temperature, and the pH of the mobile phase was studied, and their slight variation did not affect the separation of the investigated substances as described under robustness (cf. **Figure 4**).

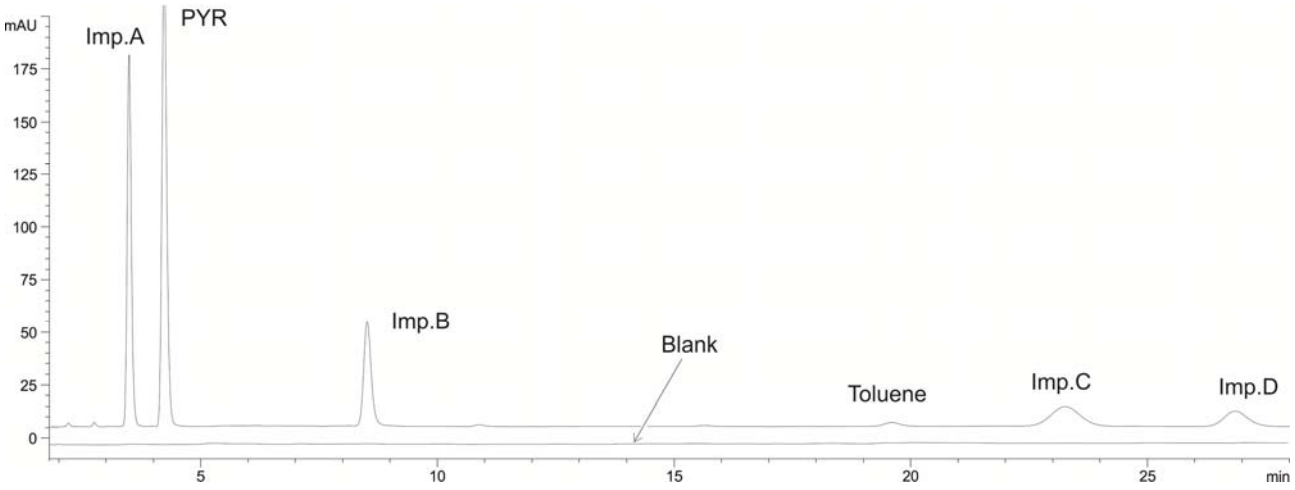
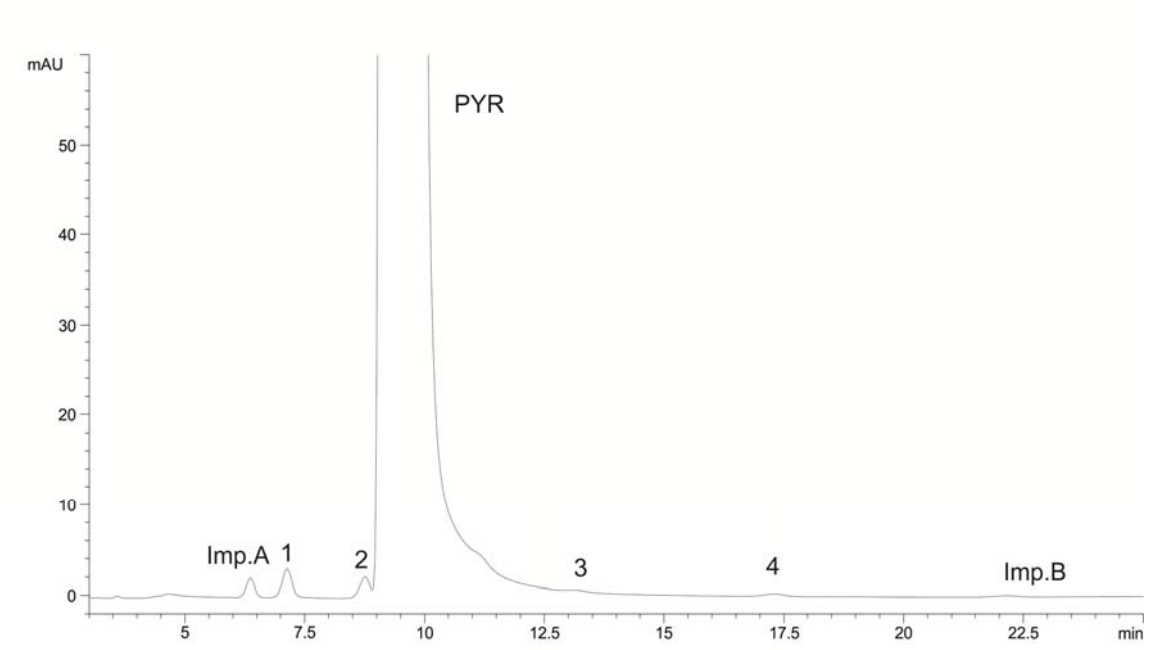


Figure 2: Chromatogram showing the separation of pyrimethamine from its impurities using the optimized method.

Method (A)



Method (B)

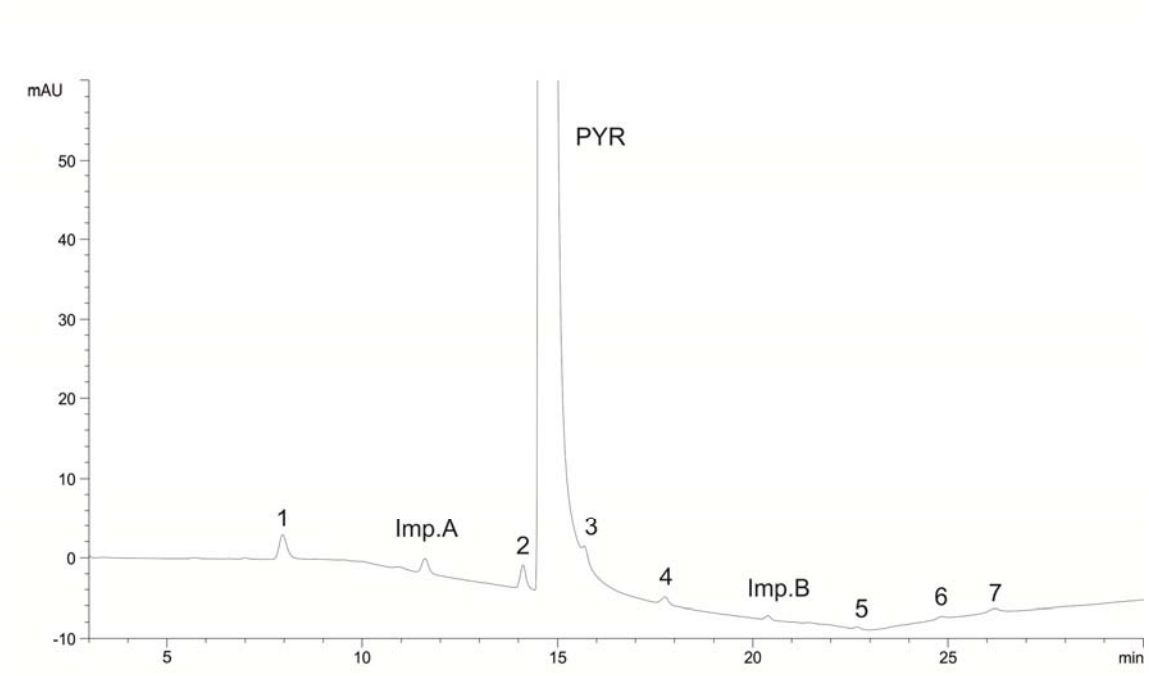


Figure 3: Chromatogram showing the separation of unknown pyrimethamine impurities of a genuine sample (1-7) using the optimized method (A) and the manufacturer method (B).

3.2 Specificity

No signals in the blank injections coeluted or interfered with the signals due to pyrimethamine and its respective impurities. All related compounds were resolved from the API; the lowest R_s value was observed to be 1.5 between impurity C and D.

3.3 Linearity of the calibration line

The method proves to be linear within the concentration ranges of 0.12 – 0.740, 0.104 – 0.621, 0.120 – 0.710, 2.0 – 11.8, and 1.01 – 5.80 µg/mL for pyrimethamine, impurity A, impurity B, impurity C and impurity D, respectively. The coefficients of determinations (R^2) for the five analytes were greater than 0.994 for pyrimethamine and all impurities (see **Table 1**). The lack–of–fit test results for all substances, F calculated (F_{calc}) were less than 0.0538 calibration data which was smaller than F critical (F_{crit}) [F_{tab} (α = 0.05; df_1 = 2; df_2 = 15)] = 3.682.

3.4 Accuracy and precision

The intra-day accuracy ranged from 89.1 – 105.1, 94.5 – 102.3, 96.0 – 103.6%, 94.0 – 103.8, and 98.4 – 104.1% for pyrimethamine, impurity A, impurity B, impurity C, and impurity D, respectively (cf. **Table 2**)**.** The acceptable limits for recovery and variation were 85 – 115% and 0 – 15% RSD [15, 16].

Repeatability precision for pyrimethamine ranged from 89.1 – 99.3, 94.5 – 99.0, 98.0 – 103.7%, 100.0 – 103.8, and 98.4 – 104.1% for pyrimethamine, impurity A, impurity B, impurity C, and impurity D, respectively. The % RSD ranged from 0.2 – 4.3% for all substances (cf. **Table 3**). All values were within the acceptable limits of 85 – 115% and 0 – 15% RSD [15, 16]. Thus, the method was found to be precise and accurate.

Table 1: Linearity study results of pyrimethamine and its impurities

Target level (%)	Amount taken from stock solution (mL)	Final Conc. (µg/mL)	Target level (%)	Amount taken from stock solution (mL)	Final Conc. (µg/mL)
Pyrimethamine			Impurity A		
0.03	0.5	0.120	0.02	0.5	0.104
0.05	1.0	0.250	0.04	1.0	0.210
0.07	1.5	0.400	0.06	1.5	0.311
0.10	2.0	0.500	0.08	2.0	0.414
0.12	2.5	0.620	0.10	2.5	0.520
0.15	3.0	0.740	0.12	3.0	0.621
Slope	154312.416 ± 32049.038		Slope	134175.320 ± 28150.122	
y-intercept	5.102 ± 2.861		y-intercept	2.496 ± 0.581	
R^2	0.995 ± 0.002		R^2	0.998 ± 0.0009	
Fcalc	0.00467		Fcalc	0.00467	
Correction Factor (CF)	N/A		Correction Factor (CF)	N/A	
Impurity B			Impurity C		
0.024	0.2	0.200	0.4	0.4	2.000
0.050	0.5	0.500	0.8	0.8	3.920
0.070	1.0	1.000	1.2	1.2	6.000
0.100	1.5	1.500	1.6	1.6	7.800
0.120	2.0	2.000	2.0	2.0	9.800
0.140	2.5	2.500	2.4	2.4	11.800
Slope	98890.100 ± 1966.782		Slope	55948.667 ± 533.146	
y-intercept	2.390 ± 1.386		y-intercept	15.858 ± 4.047	
R^2	0.996 ± 0.0015		R^2	0.994 ± 0.001	
Fcalc	0.0538		Fcalc	0.000105	
Correction Factor (CF)	1.56		Correction Factor (CF)	2.76	
Impurity D					
0.2	0.20	1.010			
0.4	0.40	2.020			
0.6	0.60	3.040			
0.8	0.80	4.050			
1.0	1.00	4.820			
1.2	1.20	5.800			
Slope	33991.300 ± 167.432				
y-intercept	30.173 ± 0.352				
R^2	0.997 ± 0.0006				
Fcalc	0.00139				
Correction Factor (CF)	4.54				

Table 2: Recovery results for pyrimethamine and its impurities.

Substance name	Target level (%)	Added amount (µg/mL; *n* = 15)	Recovered amount (µg/mL; *n* = 15)
Pyrimethamine	0.03	0.127	0.113 (89.1% ± 3.722 (4.2%)
	0.05	0.255	0.268 (105.1% ± 5.900 (5.6%)
	0.07	0.381	0.385 (100.7% ± 3.900 (3.8%)
	0.10	0.510	0.500 (98.1%± 2.894 (3.0%)
	0.12	0.637	0.636 (99.8%± 1.000 (1.0%)
	0.15	0.765	0.759 (99.3% ± 3.536 (3.6%)
Impurity A	0.02	0.104	0.111 (94.5% ± 2.600 (2.7%)
	0.04	0.207	0.248 (102.3% ± 3.100 (3.0%)
	0.06	0.311	0.342 (98.3% ± 1.300 (1.3%)
	0.08	0.414	0.420 (98.7 ± 0.500 (0.5%)
	0.10	0.518	0.558 (100.7 ± 1.500 (1.5%)
	0.12	0.621	0.630 (99.0 ± 0.300 (0.3%)
Impurity B	0.024	0.119	0.124 (103.7 % ± 4.435 (4.3%)
	0.050	0.238	0.228 (95.9 % ± 2.200 (2.0%)
	0.070	0.357	0.362 (101.3 % ± 1.794 (1.8%)
	0.100	0.476	0.466 (98.0 % ± 2.261 (2.3%)
	0.120	0.595	0.616 (103.6 % ± 0.926 (0.9%)
	0.140	0.714	0.714 (100.1 % ± 2.293) (2.3%)
Impurity C	0.4	1.960	2.030 (103.8 % ± 0.864 (0.8%)
	0.8	3.920	3.680 (94.0 % ± 0.770 (0.8%)
	1.2	5.880	6.248 (106.3 % ± 0.910 (0.9%)
	1.6	7.840	7.830 (100.0 % ± 0.614 (0.6%)
	2.0	9.800	9.456 (96.5 % ± 0.195 (0.2%)
	2.4	11.760	11.930 (101.5 % ± 0.243) (0.2%)
Impurity D	0.2	1.011	1.052 (104.1 % ± 1.061 (1.0%)
	0.4	2.022	2.028 (100.3 % ± 0.300 (0.2%)
	0.6	3.032	3.013 (99.4 % ± 0.200 (0.2%)
	0.8	4.044	3.977 (98.4 % ± 0.235 (0.2%)
	1.0	4.813	4.921 (102.2 % ± 0.200 (0.2%)
	1.2	5.784	5.724 (99.1 % ± 0.250) (0.3%)

Table 3: Repeatability studies for pyrimethamine and its impurities.

Substance name	Target level (%)	Retention time (min) (*n* = 9)	Added amount (µg/mL; *n* = 9)	Recovered amount (µg/mL; *n* = 9)
Pyrimethamine	0.03	4.5 ± 0.060 (0.1%)	0.127	0.113 (89.1% ± 3.722 (4.2%)
	0.10	4.5 ± 0.042 (0.9%)	0.510	0.500 (98.1%± 2.894 (3.0%)
	0.15	4.4 ± 0.014 (0.3%)	0.765	0.759 (99.3% ± 3.536 (3.6%)
Impurity A	0.02	3.6 ± 0.004 (0.1 %)	0.104	0.111 (94.5% ± 2.600 (2.7%)
	0.08	3.6 ± 0.014 (0.2%)	0.414	0.420 (98.7 ± 0.500 (0.5%)
	0.12	3.6 ± 0.008 (0.2%)	0.621	0.630 (99.0 ± 0.300 (0.3%)
Impurity B	0.24	9.1 ± 0.087 (0.9%)	0.119	0.124 (103.7 % ± 4.435 (4.3%)
	0.1	9.2 ± 0.065 (0.7%)	0.476	0.466 (98.0 % ± 2.261 (2.3%)
	0.14	9.1 ± 0.088 (1.0%)	0.714	0.714 (100.1 % ± 2.293) (2.3%)
Impurity C	0.4	27.0 ± 0.162 (0.6%)	1.960	2.030 (103.8 % ± 0.864 (0.8%)
	1.6	27.1 ± 0.224 (0.8%)	7.840	7.830 (100.0 % ± 0.614 (0.6%)
	2.4	27.0 ± 0.019 (0.1%)	11.760	11.930 (101.5 % ± 0.243) (0.2%)
Impurity D	0.2	29.4 ± 0.022 (0.1%)	1.012	1.052 (104.1 % ± 1.061 (1.0%)
	0.8	29.4 ± 0.000 (0.0%)	4.049	3.977 (98.4 % ± 0.235 (0.2%)
	1.2	29.5 ± 0.000 (0.0%)	5.784	5.724 (99.1 % ± 0.250) (0.3%)

3.5 Stability of standard and sample solutions

Impurity D standard solution was concluded to be stable up to 48 h while all other solutions including the sample solutions were unstable because a decrease of the peak areas and additional peaks were observed. The changes from initial peak areas were higher than the limit of ± 2.0% [15, 16] **(**cf. **Table 4).** Therefore, all solutions need to be freshly prepared.

Table 4: Stability of standard and sample solutions.

	Peak area [mAU](±2.0)		
Substance Name	Initial	24 h (Δ%)	48 h (Δ%)
Standard solution			
Impurity A	14.314	13.980 (- 2.3%)	14.340 (- 0.2%)
Pyrimethamine	154.057	150.543 (+ 2.3%)	140.314 (+ 9.0%)
Impurity B	13.865	11.976 (+ 13.5%)	15.898 (- 14.7%)
Impurity C	127.663	131.743 (- 3.2%)	125.111 (+ 2.0%)
Impurity D	99.105	99.105 (+ 0.0%)	99.105 (+ 0.0%)
Sample solution			
Impurity A	61.000	59.210 (+ 3.0%)	55.017 (+ 9.8%)
Pyrimethamine	46456.800	45679.100 (+ 1.7%)	46157.750 (+ 0.6%)
Impurity B	5.040	5.088 (-1.0%)	4.966 (1.5%)

3.6 Robustness

The method is robust against slight changes of the percentage of methanol in the mobile phase, the column temperature (20 – 45 ºC), the flow rate (1.1 – 1.3 mL/min), and the pH of the mobile phase (2.4 – 3.0) in terms of selectivity and peak tailing factor (cf. **Figure 4**). However, temperature changes affect the retention times (impurity D R_t = 31.5 min at 20 ºC vs. 22.0 min at 45 ºC) as expected, but also resulted in a coelution of the peaks due to toluene and impurity C at 45 ºC. This was not observed at other investigated temperatures, i.e. between 20 – 40 ºC which is in line with findings reported by Wolcott et. al [17].

The back pressure was being monitored throughout method development, optimization, and validation experiments, and ranged from 250 – 255 bar which is within an acceptable range regarding the column dimensions.

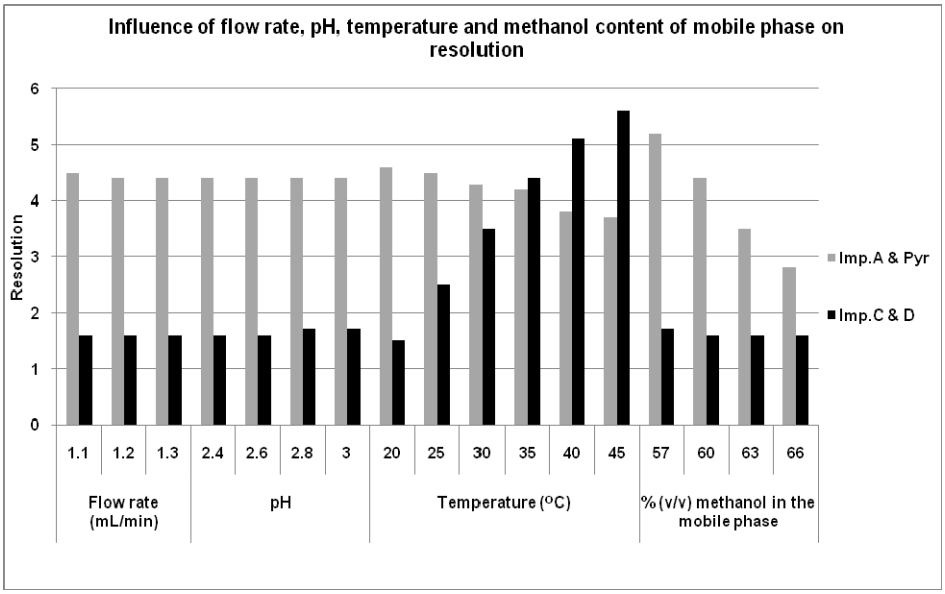


Figure 4: Robustness studies of the optimized method for pyrimethamine and its synthetic impurities.

3.7 Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD of the substances investigated ranged from 0.04 – 0.3 µg/mL, while the LOQ was 0.12 – 2.13 µg/mL (cf. **Table 5**). Pyrimethamine, impurity A, and impurity B had the lowest LOD (0.04 µg/mL) and LOQ (0.123 and 0.13 µg/mL, respectively). The percentages of impurities peak area from the main peak area ranged from 0.01 to 0.12% for LOD and from 0.013 to 0.20% for LOQ.

Table 5: Limit of Detection (LOD) and Limit of Quantification (LOQ).

Substance Name	LOD (µg/mL)	% concentration from API (500 µg/mL)	% from API Peak	LOQ (µg/mL)	% concentration from API (500 µg/mL)	% API peak area
Impurity A	0.042	0.01	0.01	0.126	0.02	0.03
Pyrimethamine	0.041	0.01	0.02	0.125	0.02	0.04
Impurity B	0.041	0.01	0.01	0.123	0.04	0.03
Impurity C	0.700	0.15	0.15	2.130	0.39	0.26
Impurity D	0.330	0.07	0.03	1.000	0.20	0.14

3.8 Related substances/synthetic impurities testing

Three batches (1, 2, and 3) of pyrimethamine were investigated for the presence of the synthetic impurities using the optimized method as well as the protocol for pyrimethamine bulk drugs provided by the manufacturer. The batches mainly contained the two specified synthetic impurities (A and B) in addition to five unspecified impurities. This observation is concurrent with results obtained from using the manufacturer's method. All impurities were within the specified limits of the manufacturer. The correction factor for each impurity was

also established and should be applied in calculation for impurity B, C, and D as their Relative Response Factor (RRF) was not within the limit i.e. 0.8 – 1.2 (the RRFs were 0.87, 0.64, 0.36, and 0.22 for Imp.A, Imp. B, Imp. C, and Imp. D, respectively) (cf. **Table 6**) [2, 6, 13]. Furthermore, statistical analysis of the two sets of results by *t*-test: Paired Two Sample for Means at a level of 0.05% indicated that there is no significant difference between results obtained by the manufacturer and developed methods for both impurities. The samples were analyzed in triplicate (duplicate injection) for each method. However, all three batches of pyrimethamine contained an unknown impurity at a concentration of approximately 0.10 µg/mL. The unknown peak eluting after impurity A was investigated by electrospray mass spectrometry and was observed to be a mixture of six compounds giving fragment ions at m/z 242.8, 304.1, 755.7, 903.5, 1385.3, and 1610.9 (pyrimethamine: 249.6). Therefore, further investigation is required to carry out a structural elucidation.

Table 6: Comparison of impurity contents obtained from test solutions of the three batches tested using the developed (A) and the current manufacturer method (B).

Batch No.			1		2		3		Limits
	Retention time (min)		[% content] (n=3)		[% content] (n=3)		[% content] (n=3)		[% content]
Method	A	B	A	B	A	B	A	B	
Unspecified impurities	NA	NA	≤ 0.1	≤ 0.1	≤ 0.1	≤ 0.1	≤ 0.1	≤ 0.1	0.1
Imp.A	3.7	11.6	0.1	0.02	0.1	0.02	0.11	0.02	0.1
Pyrimethamine	4.4	14.8	NA		NA		NA		NA
Imp.B	9.2	20.4	0.02	0.03	0.02	0.03	0.03	0.04	0.1
Total impurities	NA	NA	0.20	0.23	0.20	0.26	0.20	0.28	0.5

4. Conclusion

The developed method is very simple, but still accurate, precise, and robust against slight changes of the pH, flow rate, column temperature, and percentage of methanol content in the mobile phase. The method offers some advantages compared to compendial monographs because it can be used for the simultaneous determination of the pyrimethamine content and for impurity profiling. It is therefore considered a suitable alternative to current compendial monographs of pyrimethamine in the Ph. Eur., B. P., USP, and Ph. Int. The method can also be used for assay testing of pyrimethamine in finished dosage forms whenever applicable.

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The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interest

The authors declare that they have no competing interests.

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Authors' contributions

YHM, LH, and UH designed the study, YHM performed sample preparation and analysis, data collection, data analysis and interpretation. YHM and LH drafted the manuscript. UH and LH supervised the study and contributed in data interpretation. UH, LH, and EK reviewed of the manuscript. All authors have read and approved the final manuscript.

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3.4 Development and Validation of Simplified High-Performance Thin-Layer Chromatography Densitometric Methods for the Determination of Sulfalene, Sulfadoxine, and Pyrimethamine in Tablet Formulation

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Abstract

Background: Sulfalene, sulfadoxine, and pyrimethamine are useful active pharmaceutical ingredients in malaria treatment. Current compendial monographs and published reports for the separation and quantitative determination of the compounds mostly apply high performance liquid chromatography (HPLC) and high performance thin layer chromatography (HPTLC). However, both techniques require expensive consumables and highly purified solvents which might not be affordable for laboratories in developing countries. This warranted the development of a simple, precise, and accurate method utilizing only inexpensive thin-layer chromatographic (TLC) plates as well as safe and readily available solvents like methanol, toluene, or ethyl acetate.

Methods: Sulfalene, sulfadoxine, and pyrimethamine were determined using standard TLC plates and a mobile phase consisting of a mixture of toluene, ethyl acetate, and methanol in the ratio 50 : 28.5 : 21.5 (% v/v/v), densitometric evaluation was carried out by scanning the plates at a wavelength of λ = 254 nm.

Results: The method was validated with respect to specificity, linearity, precision, and accuracy. Linearity was proven in a concentration range of 250 - 750 µg/mL for sulfalene/sulfadoxine and 13 - 38 µg/mL for pyrimethamine. Application of the method to randomly collected marketed brands of sulfadoxine/sulfalene and pyrimethamine tablets gave content results that were in good agreement with results obtained from compendial monograph testing.

Conclusion: The developed method can be used as a basic alternative to compendial HPLC methods in the determination of the studied substances.

Keywords: counterfeit and substandard medicines; planar chromatography; densitometry; quality control; malaria

1. Background

A severe malaria outbreak in Uganda claimed 162 deaths and 22,000 cases in 2015 [1] demonstrating that this infectious disease still remains a critical topic of public health in tropical and subtropical countries. Besides the commonly known clinical symptoms, pregnant women represent one of the most vulnerable groups affected and are always at risk of developing severe malaria-related complications such as anemia, stillbirth, and premature delivery [2]. In addition to first-line treatments such as Artemisinin-based Combination Therapies (ACTs), the World Health Organization (WHO) recommends using a fixed-dose combination of a sulfonamide, e.g. sulfadoxine or sulfalene, and pyrimethamine (see **Figure 1a**) for the intermittent preventive treatment during pregnancy [3, 4]. Combining these two types of antiplasmodial active ingredients results in a synergistic effect which is due to the interference with the tetrahydrofolate and the dihydrofolate reductase enzymes in the malaria parasites [5]. Due to the wide distribution and the huge turnover of these kinds of medicines, antimalarials are often a subject of counterfeiting activities or are sold in very poor qualities which multifactorially endanger the patients and are responsible for the formation of resistances.

The compounds can be analyzed by applying a variety of techniques and methods, e.g. for sulfalene/sulfadoxine and pyrimethamine several protocols applying high performance liquid chromatography (HPLC) are described [1-9]. However, thin layer chromatography (TLC) [10] or high-performance thin-layer chromatography (HPTLC) are still being widely utilized [11, 12]. HPLC remains the most important analytical technique within this ensemble; however, massive investments in terms of delicate and expensive instrumentation, a sophisticated laboratory setup, specialized operator skills, extremely pure chemicals, and a variety of consumables are required. TLC and HPTLC represent quite simple methods being suitable for the application in resource-constrained countries or in basic laboratories [10, 13]. Hence, these planar chromatographic techniques still are widely applied [14-16]. Despite published

methods for the determination of sulfadoxine and pyrimethamine in tablets which make of use standard TLC plates, the carcinogenic solvent chloroform is added to the mobile phase or the use of haloperidol as an internal standard is reported [11]. Another method also describes a mixture of chloroform and methanol as the mobile phase, and applies expensive HPTLC plates for the simultaneous determination of sulfalene, sulfadoxine, and pyrimethamine [12].

This warranted a need to develop a simple, precise, and accurate thin-layer chromatography for simultaneous determination of sulfadoxine/sulfalene and pyrimethamine coupled with densitometric evaluation using standard TLC plates, as well as safe and readily available inexpensive solvents like methanol, toluene, and ethyl acetate as mobile phase [17]. The method can also be used in place of sophisticated and expensive HPLC methods, particularly in restricted laboratory setups where TLC or HPTLC equipment is available and liquid chromatography could not be established so far. In this paper, we developed an easy to perform planar chromatographic method and validated for the analysis of proprietary medicines containing either a fixed dose combination of 500 mg of sulfadoxine and 25 mg of pyrimethamine, or 500 mg of sulfalene and 25 mg of pyrimethamine, respectively.

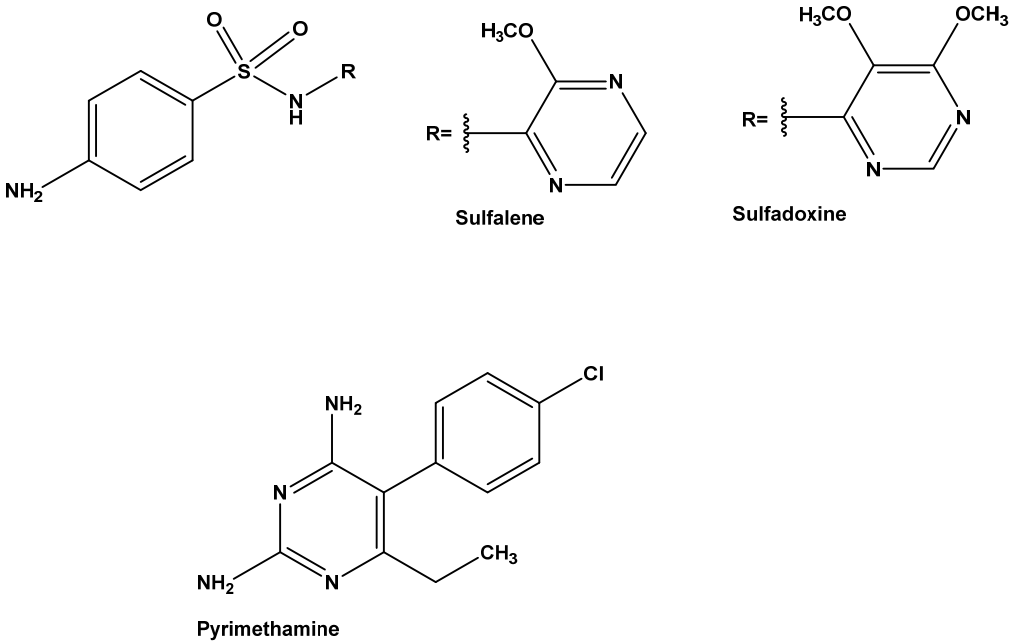

Figure 1: Chemical structural formulae of sulfadoxine, sulfalene, and pyrimethamine.

2. Methods

2.1 Materials, chemicals, and equipment

Pre-coated TLC silica plates (60 $F_{254,}$ aluminium, 20 × 10 cm) were used for method development and validation (all Merck, Darmstadt, Germany). A CAMAG HPTLC system coupled with a ‘’Linomat’’ semi-automatic applicator, a developing tank, a CAMAG TLC scanner III, WinCATS-software (version 1.4.3) as data integrator, and a Hamilton syringe of 100 µL capacity for sample application (all CAMAG, Muttenz, Switzerland) was used. Other instruments included an Ultrasonic bath from Wagtech International LTD (Berkshire, United Kingdom), and analytical balances from OHAUS Corporation (OHAUS, Pine Brook, NJ, USA) and from Mettler-Toledo (Greifensee, Switzerland). Toluene was procured from Loba Chemie Pvt. Ltd (Mumbai, India), methanol from Scharlau S.L. (Sentmenat, Spain), and ethyl acetate from Carlo Erba Reagents (Rodano, Italy). Pyrimethamine (PYR) and sulfadoxine (SD) reference standards were procured from the European Directorate for the Quality of Medicines and HealthCare (EDQM, Strasbourg, France), and sulfalene (SL) was procured from Tokyo Chemical Industry Deutschland GmbH (Eschborn, Germany).

The following generic versions of fixed dose combinations of sulfalene /pyrimethamine and sulfadoxine/pyrimethamine were collected from the local market in Tanzania and originated from both Tanzania and Kenya: *Sulphadar* (SD/PYR; B.No.140001, Shelys Pharmaceuticals LTD, Dar es Salaam, Tanzania); *Orodar* (SD/PYR; B.No. 4A05, Elys Chemical Industries LTD, Nairobi, Kenya); *Paludar-Z* (SD/PYR; B.No.TH3003, Zenufa Laboratories LTD, Dar Es Salaam, Tanzania); *Ekelfin* (SL/PYR; B.No.3D81, Elys Chemical Industries LTD, Nairobi, Kenya); *Laefin* (SL/PYR; B.No.61206, Laboratory & Allied LTD, Nairobi, Kenya); and *Malafin* (SL/PYR; B.No.130020, Shelys Pharmaceuticals LTD, Dar es Salaam, Tanzania).

2.2 Solutions

2.2.1 Mobile phase

Volumes of 50.0 mL of toluene, 28.5 mL of ethyl acetate, and 21.5 mL of methanol were mixed. The developing chamber was saturated for 25 min before developing the plates.

The diluent for stock sample and standard solution preparation was obtained by mixing

methanol and ethyl acetate in the ratio 1:1 (% v/v).

2.2.2 Plate development

Each TLC aluminum plate was pre-washed using a mixture of toluene and methanol in the ratio 1:1 by the ascending technique up to a migration distance of at least 60 mm and was subsequently dried in an oven at 105 ºC for 5 min. Samples of 4.0 µL were applied per solution at a position of 15 mm side and 10 mm bottom margin. Development of the plates was carried out by the ascending technique, and they were subsequently allowed to dry for at least 10 min. Densitometric evaluation was performed by scanning plates at λ = 254 nm using a deuterium lamp (slit dimensions: 4.00 mm × 0.30 mm, scanning speed: 20 mm/s, data resolution: 100 µm/step).

2.2.3 Sulfalene/sulfadoxine and pyrimethamine stock standard solution

A portion of 25.0 mg of pyrimethamine was dissolved in 1.0 mL of methanol, sonicated for 10 min, and diluted to 10.0 mL using the diluent. Amounts of 50.0 mg of each sulfadoxine and sulfalene were individually dissolved in 2.0 mL of methanol and sonicated for 10 min.

A volume of 1.0 mL of pyrimethamine standard solution was added to each solution and the volume was made to 20.0 mL using the diluent in order to obtain a stock standard solution containing 2.5 mg/mL of each sulfalene/sulfadoxine and 0.125 mg/mL of pyrimethamine. This procedure maintained an API concentration ratio of 20:1 which is in accordance with the amounts of sulfalene/sulfadoxine to pyrimethamine used in the respective tablet formulations.

2.2.4 Sulfalene/sulfadoxine and pyrimethamine sample solution

20 tablets of each proprietary product were weighed and ground to fine powder. An amount of powder equivalent to 250.0 mg sulfalene or sulfadoxine was suspended in 10 mL of methanol and sonicated for 10 min. The solutions were diluted to 100.0 mL using mixture of methanol and ethyl acetate (1:1) and were passed through 0.45 µm syringe filters. 2.0 mL of the filtrate were subsequently diluted to 10.0 mL using the same solvent to obtain a final solution containing 0.50 mg/mL of sulfalene or sulfadoxine, respectively, and 0.025 mg/mL of pyrimethamine.

2.3 Method development

Besides the conditions described in the Minilab® manual (i.e., ethylacetate, acetone, and glacial acetic acid - 18 : 4 : 0.1, % v/v/v) [18], mixtures of chloroform and methanol (12 : 0.5, % v/v) [12] as well as of chloroform, butanol, and acetone (6 : 1.5 : 2, % v/v/v) [11] were applied as the mobile phase. Another investigated composition included the mixture of toluene, ethyl acetate, and methanol (50 : 28.5 : 21.5, % v/v/v) which was being used previously for the determination of sulfamethoxazole [19].

2.4 Method validation

The method was validated for linearity, precision, specificity, and accuracy as per ICH guidelines [20].

2.4.1 Specificity

The specificity of the method was investigated by preparing a sample and a standard solution at a nominal concentration of 500 µg/mL of sulfalene/sulfadoxine and 25 µg/mL of pyrimethamine, which were simultaneously applied on the TLC plate. The specificity of the method was assessed by comparing the densitogram and R_f values obtained from standard solutions against those of sample solutions.

2.4.2 Linearity of the calibration line

A stock standard solution containing 2500 µg/mL of each sulfalene/sulfadoxine and 125 µg/mL of pyrimethamine was prepared and diluted to obtain five calibration solutions at levels of 250, 375, 500, 625, and 750 µg/mL for sulfalene/sulfadoxine, and 13, 19, 25, 31, and 38 µg/mL for pyrimethamine **(Table 1)**. Solutions were applied in duplicate and the whole analytical procedure was repeated on three consecutive days. The linearity was evaluated visually from plotted calibration data, and statistically by performing an F-test for lack-of-fit (LOF) which examines whether the regression line adequately fits the data.

2.4.3 Precision

Repeatability and intermediate precision using six sample solutions for each sulfadoxine/pyrimethamine and sulfalene/pyrimethamine were investigated at the nominal concentration of 500 µg/mL for sulfalene/sulfadoxine, and 25 µg/mL for pyrimethamine.

Intermediate precision was performed by a different analyst conducting analysis on three consecutive days.

2.4.4 Accuracy

Accuracy was studied by standard addition of a known amount of the three APIs to a tablet sample solution at concentration levels of 80, 100, and 120% of the assay concentration in triplicate and evaluating the recovery of the analyte at each level. [21].

3. Results and Discussion

3.1 Method development

The aim of the study was to develop a simple method for the separation and simultaneous determination of sulfadoxine, sulfalene, and pyrimethamine.

A mobile phase consisting of ethyl acetate, acetone, and glacial acetic acid (18 : 4 : 0.1, % v/v) [18] did not give any separation at all; using a mixture of chloroform and methanol (12 : 0.5 % v/v) [12] resulted in the co-elution of the solvent front and sulfalene/sulfadoxine. The mixture of chloroform, butanol, ethyl acetate, and glacial acetic acid (6 : 2 : 2 : 0.5, % v/v/v/v) gave higher R_f values for sulfadoxine/sulfalene ($R_f \geq 0.85$) and lower ones for pyrimethamine ($R_f \geq 0.17$), the densitograms also had a bumpy baseline. The mixture of chloroform, butanol, and acetone (6 : 1.5 : 2, % v/v/v) [11] was able to separate all three substances, however, pyrimethamine had a relatively high R_f value of 0.950 whereas the one for sulfadoxine was below 0.33. The densitogram particularly that of sulfadoxine, was poor due to a noisy baseline. Further attempts to improve the separation by increasing the R_f of sulfadoxine, and thus improving the quality of the sulfadoxine densitogram through adjustment of the composition of the mobile phase were not successful.

Utilizing the mixture of toluene, ethyl acetate, and methanol (50 : 28.5 : 21.5, %v/v/v) [19] using HPTLC and TLC plates gave good results. However, using the latter resulted in the best separation and the quality of the densitogram was good **(Figure 2).** The scanning wavelength of $\lambda = 254$ nm was chosen based on reported methods [11, 12, 19].

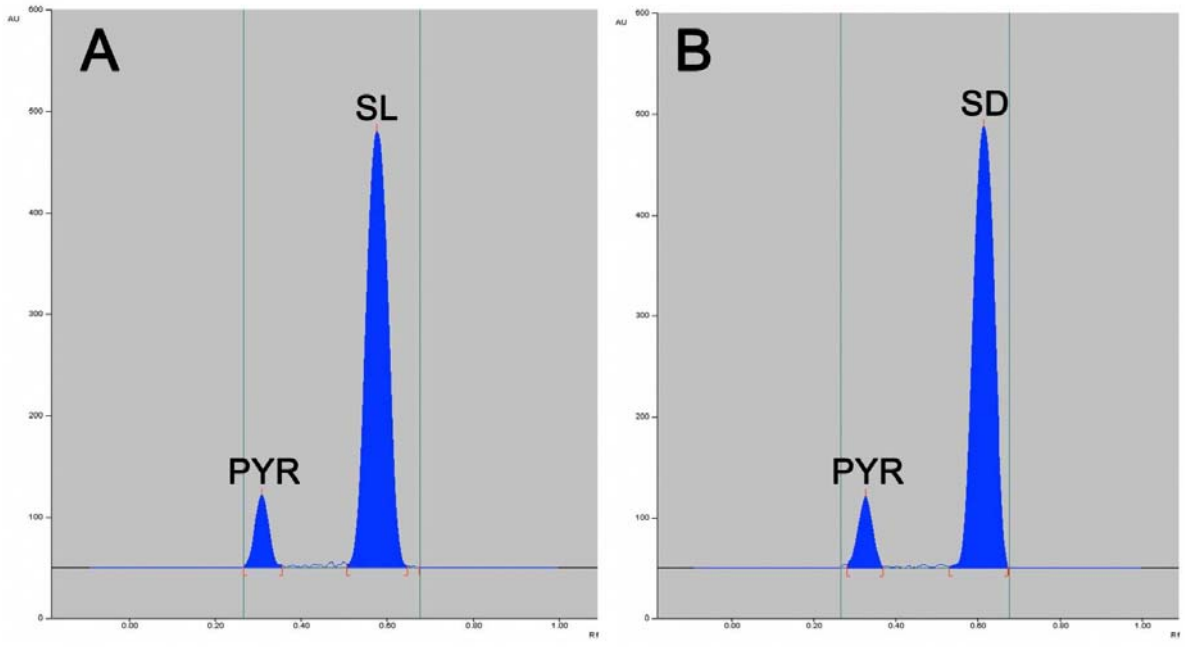

Figure 2: Densitograms showing the separation of A: sulfalene (SL) and pyrimethamine (PYR) and B: sulfadoxine (SD) and pyrimethamine (PYR) using the optimized method.

Although these mobile phases achieved the separation of either sulfadoxine or pyrimethamine or sulfalene and pyrimethamine, none was able to simultaneously separate all three substances. Both sulfadoxine and sulfalene had very similar R_f values. This is not a problem since the two substances are not formulated together, thus they can be determined separately. However, this may be a restriction for substitute detection, e.g. in the case of API exchanges. The proposed developed method utilizes standard TLC plates, harmless and readily available inexpensive methanol, toluene, and ethyl acetate as mobile phase [17] contrary to reported methods which require carcinogenic chloroform for preparing the mobile phase as well as expensive HPTLC plates [11, 12].

3.2 Method Validation

Optimum conditions were eventually found utilizing a standard thin-layer chromatographic plate and a mobile phase consisting of a mixture of toluene, ethyl acetate, and methanol in the ratio 50 : 28.5 : 21.5 (% v/v/v). Densitometric evaluation was performed by scanning the plates at λ = 254 nm.

3.2.1 Specificity

No signal(s) from the diluent, the mobile phase, or the sample solution co-eluted or interfered with the three APIs. The sample solution as well as that of standard solution, showed clear, and well-separated densitogram of sulfalene/sulfadoxine and pyrimethamine which suggest a specific method.

3.2.2 Linearity of the calibration line

The method was linear in the concentration range of 250 - 750 µg/mL and 13 – 38 µg/mL for sulfalene/sulfadoxine and pyrimethamine, respectively. The linear regression coefficient R^2 ranged from 0.98 – 0.99 for all three substances **(Table 1)**. The lack-of-fit test result calibration data was F_{calc} = 0.366 for sulfadoxine, 0.0191 for sulfalene, and 0.0417 for pyrimethamine, values which were significantly smaller than the critical value, F_{tab} = 3.490 (α = 0.05; df1 = 3, df_2 = 15). This indicates an adequate linear relationship between the peak area and the concentration for each compound.

Table 1: Linearity studies for sulfadoxine/sulfalene (SD/SL) and pyrimethamine (PYR).

Level (%)	Stock solution (mL)	matrix added (mL)	Final volume (mL)	Final SD conc. (µg/mL)	Final SL conc. (µg/mL)	Final PYR conc. (µg/mL)
50	1	1	10	250	250	13
80	3	1	20	375	375	19
100	1	1	5	500	500	25
120	5	1	20	625	625	31
150	3	1	10	750	750	38
Slope				15294x (±2052.761)	15075.30x (±763.005)	49813.3x (±9510.98) 53764.67x (±2073.141)
y-intercept				5435 ±1516.803	5541.17 ± 459.383	153.053 ± 108.786 145.670 ± 22.60
R^2				0.992 ± 0.004	0.990 ± 0.009	0.982 ± 0.012 0.997 ± 0.004

2.3.3 Precision

Repeatability ranged from 0.9, 3.5, and 0.0 - 2.4% rsd for sulfadoxine, sulfalene, and pyrimethamine, respectively. Intermediate precision ranged from 2.4, 2.3 and 2.3 – 3.7% rsd for sulfadoxine, sulfalene, and pyrimethamine, respectively **(Table 2)**.

Table 2: Repeatability and intermediate precision of sulfalene (SL), sulfadoxine (SD), and pyrimethamine (PYR).

	Repeatability (% Recovery & R_f)							
	SD	R_f	PYR	R_f	SL	R_f	PYR	R_f
Mean	102.8	0.7	98.4	0.4	94.45	0.7	91.2	0.4
sd	0.919	0	2.404	0.000	3.323	0	0	0
rsd	0.9	0	2.4	0	3.5	0	0	0
n	12	12	12	12	12	12	12	12
	Intermediate							
	SD	R_f	PYR	R_f	SL	R_f	PYR	R_f
Mean	107.6	0.7	94.1	0.4	99.5	0.745	96.5	0.5
sd	2.546	0.000	2.121	0.000	2.263	0.007	3.536	0.000
rsd	2.4	0.0	2.3	0.0	2.3	0.9	3.7	0.0
n	6	6	6	6	6	6	6	6

3.2.4 Accuracy

The accuracy ranged from 96.3 – 100.6%, 97.8 – 98.0%, and 97.3 – 100.4% for sulfadoxine, sulfalene, and pyrimethamine, respectively. The overall mean recoveries for all levels were 98.0, 97.2, and 101.8% for sulfadoxine, sulfalene, and pyrimethamine, respectively **(Table 3)**. This suggests that the method has an acceptable recovery except for sulfalene. The lower amounts reported might be due to the generally poorer sensitivity of the method compared to other chromatographic techniques.

Table 3: Recovery study results of sulfalene (SL), sulfadoxine (SD), and pyrimethamine (PYR) determined in different tablet matrices.

Levels (%) (n = 9)	Average added amount (µg/mL)	Average recovered amount (µg/mL)	Average recovery (% ± sd; %rsd)
		SD	
80	375	361	96.3 ±4.055; 4.2
100	500	467	97.1 ±4.441; 4.6
120	625	629	100.6 ±6.109; 6.1
		PYR	
80	18.9	18.3	97.3 ±0.833; 0.9
100	25.2	26.4	104.8 ±0.907; 0.9
120	31.4	31.5	100.4 ±4.552; 4.5
		SL	
80	376	368	97.8 ±2.495; 2.5
100	501	480	95.8 ±2.930; 3.5
120	627	613	98.0 ±2.495; 2.5
		PYR	
80	18.9	19.3	102.1 ±1.595; 1.6
100	25.2	27.2	107.8 ±4.257; 3.9
120	31.5	31.0	98.4 ±0.115; 0.1

3.2.5 Analysis of proprietary pharmaceutical products from the Tanzanian market

The content of two samples of each sulfadoxine/pyrimethamine and sulfalene/pyrimethamine products was analyzed using the optimized method. In addition, the same samples were

analyzed applying the liquid chromatographic method from the Ph. Int. monograph [3] as well as a previously published, simplified liquid chromatography method [5]. The assay results obtained using these three different methods were compared as shown in **Table 4**.

Statistical analysis using Excel's two samples for paired two means (F- test) revealed that there was no significant difference between the sulfadoxine/sulfalene and pyrimethamine assay results obtained using the different methodologies except for one brand (Paludar-z). In the case of Paludar-z, there was no significant difference in assay results obtained using the three methods for sulfadoxine but there was a significant difference for pyrimethamine assay results. These lower contents might be reasoned by differences in formulation and the fact that the amount of pyrimethamine corresponds to only 5% of the respective sulfonamide. Thus, in this case the limited sensitivity of TLC might be problematic.

Table 4: Assay results of SL/PYR and SD/PYR in tablets obtained using different methods.

Generic Product		Ph. Int. Assay	HPLC [5]	TLC	Ph. Int. Assay	HPLC [5]	TLC
		SD			PYR		
Paludar-Z	Mean	101.2	103.4	108.7	100	102.8	93.4
(SD/PYR)	sd	0.283	0.361	2.404	0.141	0.153	0.141
	rsd	0.3	0.3	2.2	0.1	0.1	0.1
Orodar	Mean	101.9	103.1	107.6	102.6	104.1	94.1
(SD/PYR)	sd	0	0.451	2.550	0.141	0.404	2.121
	rsd	0	0.4	2.4	0.1	0.4	2.3
		SL			PYR		
Malafin	Mean	96.1	97.2	99.6	99.9	99	96.5
(SL/PYR)	sd	0	0.321	2.200	0.141	0.306	3.536
	rsd	0	0.3	2.2	0.1	0.3	3.7
Laefin	Mean	95.6	95.7	99.3	98.6	96.7	92.2
(SL/PYR)	sd	0.141	1.48	2.148	0.99	1.704	2.477
	rsd	0.1	1.5	2.2	1	1.8	2.7

4. Conclusion

The final optimized TLC method for the quantitative determination of sulfadoxine/sulfalene and pyrimethamine is simple, accurate, and precise. The use of standard thin layer chromatography plates as well as safe and readily available inexpensive solvents is anticipated to make the method to be most affordable.

The comparable assay results obtained using the optimized method as well as compendial and literature methods suggest that the method can be used as an alternative to the pharmacopoeial monograph, particularly in countries where densitometric equipment is

already available. Planar chromatography may provide a shorter analysis time and reduced costs, offers a quick sample preparation (no particle filtration, no solvent degassing), and is economic regarding a low solvent consumption and no carry-over from a previous analysis. However, the sensitivity and accuracy of the method might be limited [12, 15], taken together it is considered suitable for screening test or quick quality evaluations.

Competing interest

The authors declare no conflict of interest.

Authors' contributions

YHM, EK, and UH designed the study; YHM performed sample preparation, data collection, analysis, and interpretation of data; YHM and LH drafted the manuscript. EK and UH supervised the study. UH, DHS, LH, and EK contributed in reviewing of the manuscript. All authors read and approved the final manuscript.

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3.5 Development and Validation of a Simple and Robust Isocratic Liquid Chromatographic Method for the Determination of Primaquine and its Synthetic Impurities in Bulk Material and Pharmaceutical Formulations

Abstract

A simple, accurate, and precise RP-HPLC method was developed for the simultaneous determination of primaquine and its four synthetic impurities in bulk drugs and pharmaceutical formulations. Separation was achieved on a standard reverse phase (C_{18}) column using a mobile phase being composed of 30 % (v/v) of a 0.05 M ammonium formate buffer solution (pH = 2.7) and 70 % (v/v) of methanol, applying an isocratic elution mode and a detection wavelength of λ = 265 nm. The method was validated with respect to specificity, linearity, precision, accuracy, limits of detection and quantification, and robustness.

Keywords: primaquine**;** RP-HPLC; quality control; bulk drug and tablet analysis; malaria; impurity profiling

1. Introduction

Primaquine (8-[(4-amino-1-methylbutylamino)amino]-6-methoxyquinoline) is classified under the 8-aminoquinoline antimalarial drugs which are effective against hepatic forms of plasmodia (chemical structures see **Figure 1**). It acts through interfering in the process of electron transfer in the parasite mitochondria which results in its swelling and vacuolization because of damage to the enzymatic systems [1, 2]. Other modes of action are also discussed. However, the mechanism is not fully understood yet. It is a useful drug for prophylaxis particularly for travelers from non-endemic malaria areas such as the temperate countries [2, 3]. The current compendial monograph in the British Pharmacopoeia (B. P.) describes the determination of primaquine and its impurities by utilizing normal phase chromatography and a detection carried out at λ = 261 nm, but the respective impurity is not specified [4]. The United States Pharmacopoeia (USP) monograph describes impurities testing using liquid chromatography with a mobile phase consisting of acetonitrile, tetrahydrofuran, trifluoroacetic acid, and water as well as a C_8 column at λ = 265 nm.

Nevertheless, it also lacks a detailed description of specified impurities except for impurity A (8-(4-aminopentylamino)-6-methoxyquinoline) and secaquine [5]. The International Pharmacopoeia (Ph. Int.) depicted related substance testing by thin-layer chromatography (TLC) without specifying any impurities [6]. Other published reports explained the content determination of primaquine utilizing liquid chromatography with gradient mode elution and detection carried out at λ = 254, 260, and 331 nm [7-9], or the determination of 8-(4-amino-4-methylbutylamino)-6-methoxyquinoline and nitro-6-methoxyquinoline by gradient elution comparing HPLC and UPLC, at λ = 265 nm [10]. The lack of protocol for the determination of all known primaquine synthetic impurities warranted the development and validation of a simple, cheap, precise, and accurate HPLC method for the analysis of primaquine together with its related compounds. Utilizing easily available and inexpensive chemicals, reagents, and basic equipment is considered crucial for allowing the applicability in resource-constrained countries [11, 12]. Hence, the mobile phase consists of an ammonium formate buffer and methanol, while the stationary phase is a commercially available, inexpensive reversed phase C_{18} column.

2. Material and Methods

2.1 Materials

Primaquine, 6-methoxy-8-nitroquinoline (impurity B), 6-methoxyquinolin-8-amine (impurity C), and 2-(4-((6-methoxyquinolin-8-yl)amino)pentyl)isoindole-1,3-dione (impurity D) were obtained from the European Directorate for the Quality of Medicines and HealthCare (EDQM, Strasbourg, France).

Ammonium formate (NH_4HCO_2), methanol, and formic acid (all HPLC grade) were purchased from Sigma-Aldrich (Steinheim, Germany). Purified water for buffer preparation was generated by a Milli-Q® laboratory water system from Merck Millipore (Darmstadt, Germany).

Method development and validation were carried out by employing standard reverse phase C_{18} columns, i.e. an XTerra RP_{18} column (column A, being a polymer-bonded, reversed phase and end capped stationary phase with 15% carbon load, 250 x 4.6 mm, particle size 5

µm) (Waters, Ireland), an Eurospher-II C_{18}A column (column B; characterized by a polar end capping and a lower hydrophobicity 150 x 4.6 mm, particle size 5 µm), and an Eurospher-II C_{18}H column (column C; being polymer-bonded, highly hydrophobic and fully end capped (125 x 4.6 mm, particle size 5 µm)) (both Knauer, Berlin, Germany).

2.2 Instruments

Method development and validation were conducted using an Agilent 1100 liquid chromatographic system equipped with an on-line degasser, a binary pump, an automatic sample injector, and a variable wavelength detector. Data was acquired and processed using the Agilent ChemStation for LC 3D Systems software (Rev. B. 03.02) (341). Unless stated otherwise, a sample of 10.0 µL of each solution was injected into the chromatographic system. Other equipment included a Metrohm AG pH meter from Deutsche Metrohm Prozessalytik GmbH (Filderstadt, Germany), an ultrasonic bath from Bandelin Electronic GmbH (Berlin, Germany), and analytical balances from Sartorius AG (Göttingen, Germany) as well as Mettler Toledo (Greifensee, Switzerland). Mobile phases for liquid chromatography were passed through membrane filters (0.45 µm) prior to use. Samples, as well as standard solutions, were passed through 0.45 µm syringe filters prior to injection into the HPLC system.

2.3 Preparation of buffer solution and mobile phases

A portion of 3.129 g of NH_4HCO_2 was dissolved in 800 mL of purified water, and the pH was adjusted to 2.7 by adding formic acid. The volume was made up to 1000.0 mL with water, and the pH was verified. The mobile phase was prepared by mixing 300 mL of the buffer solution with 700 mL of methanol and sonicating the solution for 15 min.

2.4 Preparation of standard stock solutions

Samples of 5.00 mg of primaquine, 7.50 mg of impurity B, and 7.50 mg of impurity C were dissolved in 25 mL of the mobile phase, sonicated for 15 min and diluted to 50.0 mL using the mobile phase (solution A). A sample of 3.75 mg of impurity D was dissolved in 10.0 mL of methanol, sonicated for 15 min, and diluted to 50.0 mL using the mobile phase (solution B). 1.0 mL of solution A and 2.0 mL of solution B were diluted to 100.0 mL using the mobile

phase resulting in a concentration of 0.0015 mg/mL of impurities B, C, and D as well as 0.001 mg/mL of primaquine (solution C).

2.5 Preparation of sample solutions for impurity testing

A sample of 10.0 mg of each batch of primaquine diphosphate was individually dissolved in 10.0 mL of the mobile phase and sonicated for 15 min. The volume was made up to 20.0 mL using the mobile phase resulting in a concentration of 0.50 mg/mL of the primaquine.

2.6 Standard solution for impurities determination

A sample of 5.0 mL of solution C was diluted to 10.0 mL with the mobile phase resulting in a final concentration of 0.0005 mg/mL for primaquine, and 0.00075 mg/mL for impurities B, C, and D.

2.7 Method validation

Validation was conducted in accordance with the guidelines of the International Council for Harmonization (ICH) [13].

2.7.1 Specificity

Specificity of the method was studied by preparing standard solutions at working concentrations, i.e. 0.00075 mg/mL of each impurity B, impurity C, impurity D, and 0.0005 mg/mL of primaquine (containing trace amounts of impurity A), as well as 0.500 mg/mL of sample solutions of the primaquine batches. The specificity of the method was assessed by comparing the chromatograms and retention times obtained using standard solutions against that of sample solutions to ensure that none of them coeluted with the Active Pharmaceutical Ingredient (API) or with each other.

2.7.2 Linearity of the calibration line

Calibration solutions were prepared by diluting appropriate aliquots of each stock solution (cf. 2.4) to obtain six concentration corresponding to 0.008 - 0.09% of the primaquine solution having a nominal concentration of 0.5 mg/mL (cf. **Table 1**).

2.7.3 Recovery

The accuracy of the method was investigated by recovery studies carried out by the addition of a standard in which a known amount of both API and the investigated impurities were added to the mobile phase at levels of 0.008 – 0.09% of primaquine nominal concentration (0.5 mg/mL) as described in 2.7.1.2.

2.7.4 Limit of Detection (LOD) and Limit of Quantification (LOQ)

Limit of Detection (LOD) and Limit of Quantification (LOQ) were determined based on the standard deviation of the response and the slope using six concentration levels (cf. linearity). The values for LOD and LOQ were calculated as provided within the ICH guideline [13].

2.7.5 Precision: repeatability

Six independent solutions were prepared at the LOQ, at levels of 0.04%, and 0.06% of the nominal concentration for primaquine as well as at the LOQ, and two other levels for each impurity. This was investigated on three consecutive days using separately weighed amounts of the impurities and primaquine.

2.7.6 Robustness

Robustness was determined by slightly varying the chromatographic conditions including the amount of the organic solvent at ± 5% (i.e., 66.5, 70, and 73.5%), the column temperature (i.e., 25, 30, and 35 °C), and the pH of the aqueous component at ± 0.2 pH units (i.e., 2.5, 2.7, and 3.0). The flow rate was adjusted by ± 0.1 mL/min (i.e., 0.65, 0.75, and 0.85 mL/min).

2.8 Quantitative determination of primaquine synthetic impurities according to the Manufacturer method

Stationary phase: a Symmetry RP-8 HPLC column (0.15 m x 4.6 mm, 5 µm particle size) (Waters, UK). Mobile phase A: A mixture of 90 mL of acetonitrile, 10 mL of tetrahydrofuran, 1 mL of trifluoroacetic acid, and 900 mL of distilled water. Mobile phase B: acetonitrile.

Test solution: A sample of 25.0 mg of each batch of primaquine was individually dissolved in 25 mL of mobile phase A, sonicated for 10 min, and diluted to 50.0 mL with the same solvent.

Reference solution (a): 5.0 mg of primaquine phosphate was dissolved in 5 mL of mobile phase A, sonicated, and diluted to 10.0 mL with the same solvent.

Reference solution (b): 10.0 mg of primaquine phosphate, 15.0 mg of impurity B, and 15.0 mg of impurity C were dissolved in 60 mL of mobile phase A, sonicated for 10 min, and diluted to 100.0 mL with the same solvent. A portion of 0.5 mL of the reference solution (b) was diluted to 10 mL with mobile phase A.

Reference solution (c): 7.5 mg of impurity D was dissolved in 5 mL of acetonitrile and sonicated for 10 min, 75 mL of mobile phase A was added, sonicated for another 10 min, and diluted to 100.0 mL with the same solvent. A portion of 1.0 mL of reference solution (c) was diluted to 10.0 mL with the mobile phase

A. Reference solution (d): 1.0 mL of reference solution (b) and 1.0 mL of reference solution (c) were diluted to 10.0 mL with mobile phase A.

A gradient programme was employed: 0 – 15 min: 100% A; 15 – 40 min: 100 → 50% A; 40 – 45 min: 50% A; 45 – 50 min: 50 → 100% A; 50 min: 100% A. Analysis was carried out at 25 °C applying a flow rate of 1.2 mL/min and a detection wavelength of λ = 265 nm. The injection volume was 25 µL.

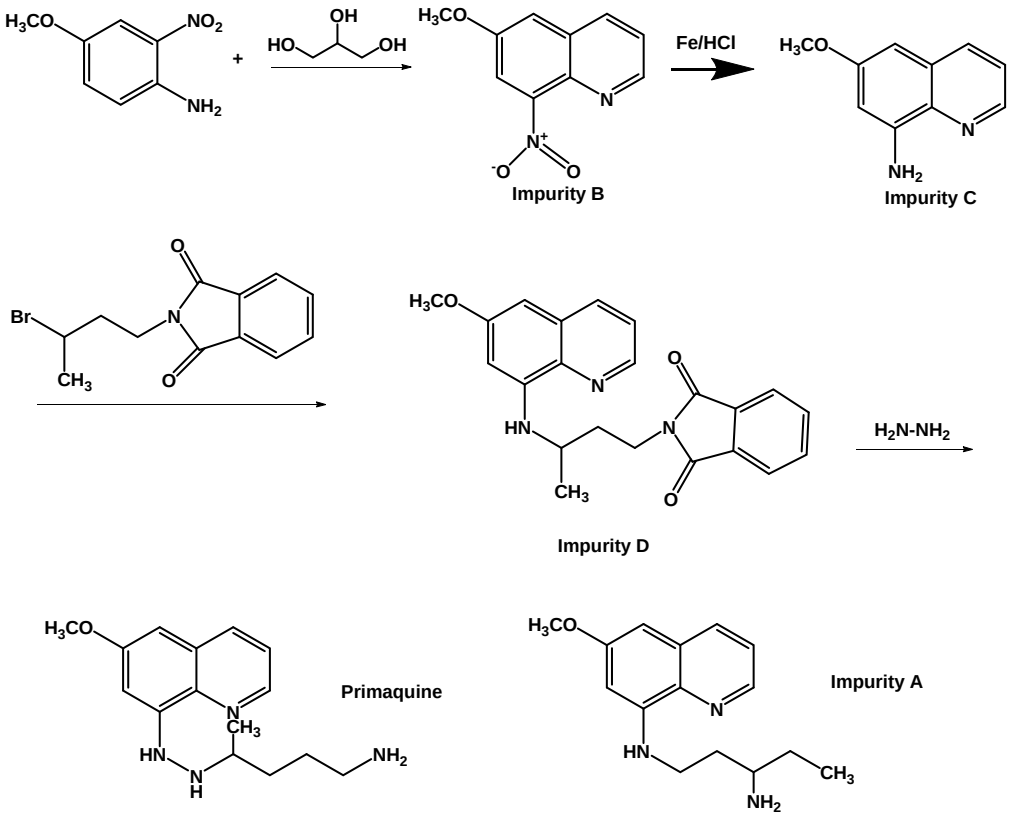

Figure 1: Primaquine synthesis pathway according to Vardanyan et.al. [1]

3. Results and Discussion

The objective of this investigation was to separate and quantify primaquine and four of its synthetic impurities: impurity A (an isomer of primaquine), impurity B (starting material), and impurities C and D (intermediate products) (**Figure 1**). It was also aimed to avoid (a) expensive solvents such as acetonitrile and tetrahydrofuran, and (b) a gradient elution. Both were applied by the manufacturer.

3.1 Method Development

The basic nature of primaquine having pK_a values of 3.2 and 10.4, warranted a 0.05 M KH_2PO_4 buffer solution adjusted to pH = 2.6 which was mixed with methanol at a range of 40 – 60% (v/v). This mobile phase was used as a starting point for the separation of all five analytes [14, 15]. However, the conditions resulted in a broad peak for impurity D. Similar results were observed utilizing Na_2HPO_4 as a buffer salt. Working at a higher percentage of methanol proved to be an option. However, due to a possible precipitation of phosphate buffers in the presence of methanol, other buffers based on glacial acetic acid, phosphoric acid, ammonium acetate, and ammonium formate were tested [16, 17]. The optimal

chromatographic conditions were eventually found by employing an XTerra RP_{18} column (250 x 4.6 mm, particle size 5 µm) and a mobile phase being a mixture of a 0.05 M NH_4HCO_2 buffer solution (pH = 2.7) with methanol in the ratio 30:70 (v/v). The analysis was carried out at 30 ºC, applying a flow rate of 0.75 mL/min, and a detection wavelength of λ = 265 nm, resulting in a total runtime of 25 min.

3.2 Validation of the method for determination of the related substances

3.2.1 Specificity

Any signal in the blank injections did not coelute or interfere with the signals due to primaquine and its respective impurities, whereby all resolutions were higher than 2.0. A typical representative chromatogram is shown in **Figures 2a-b**.

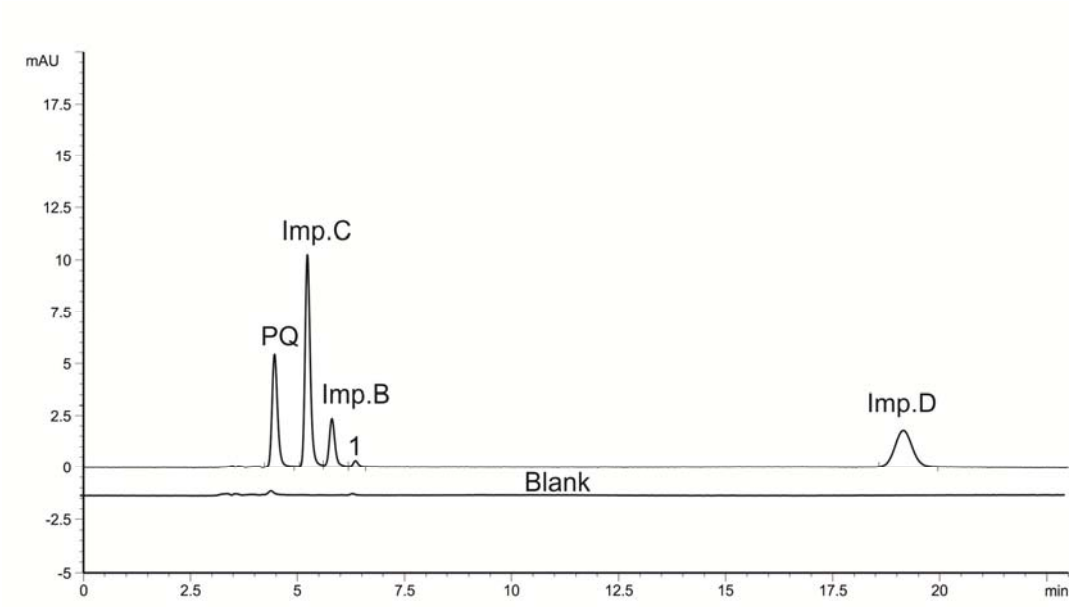

Figure 2a: Chromatogram showing the separation of primaquine (PQ) and its three impurities B, C, and D obtained from a standard solution at approx. 0.0005 – 0.0007 mg/mL using the optimized method (signal 1 is unknown).

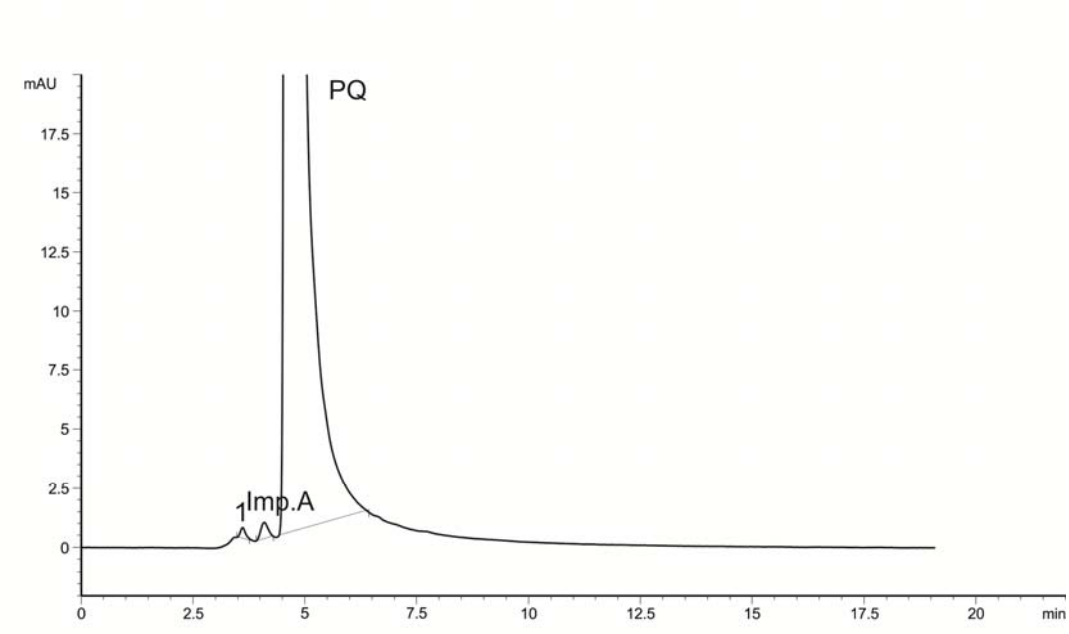

Figure 2b: Chromatogram showing the separation of primaquine (PQ) and impurity A obtained from a primaquine standard solution at 0.50 mg/mL using the optimized method (signal 1 is unknown).

3.2.2 Linearity of the calibration line

The method proves to be linear within the concentration range of 0.100 – 0.353 µg/mL for primaquine, 0.150 – 0.530 µg/mL for impurity B, 0.075 – 0.450 µg/mL for impurity C, and 0.038 – 0.228 µg/mL for impurity D. The coefficients of regression (R^2) for all the five analytes were higher than 0.993 (see **Table 1**). The Lack–of–Fit Test (LOF) results for all substances, F calculated (F_{calc}) results were less than 0.600 calibration data which was smaller than F critical (F_{crit}) [F_{tab} ($\alpha = 0.05$; $df_1 = 2$; $df_2 = 17$)] = 3.682.

Table 1: Linearity study results of primaquine and its impurities B, C, and D.

	Final conc. (µg/mL) vs (%) with respect to the test solution			
	Primaquine	Imp.B	Imp.C	Imp.D
	0.100 (0.02%)	0.150 (0.03%)	0.075 (0.02%)	0.038 (0.008%)
	0.150 (0.03%)	0.225 (0.05%)	0.150 (0.03%)	0.076 (0.015%)
	0.200 (0.04)	0.300 (0.06%)	0.225 (0.05%)	0.114 (0.023%)
	0.250 (0.05%)	0.375 (0.08%)	0.300 (0.06%)	0.152 (0.030%)
	0.300 (0.06)	0.450 (0.09%)	0.375 (0.08%)	0.190 (0.040%)
	0.353 (0.07%)	0.530 (0.1%)	0.450 (0.09%)	0.228 (0.050%)
Regression coefficient (R^2) ± SD	0.993 ± 0.003	0.998 ± 0.002	0.999 ± 0.000	0.999 ± 0.001
Intercept ± SD	1.715 ± 1.016	0.569 ± 0.033	0.769 ± 0.202	0.295 ± 0.052
Slope ± SD	49555 ± 6464.40	14243.09 ± 978.205	59376.67 ± 916.415	69480.53 ± 1527.873
Fcal	0.511	0.195	0.003	0.0124
Correction factor (CF)	NA	3.48	NA	0.713

3.2.3 Accuracy and precision

The accuracy ranged from 98.4 – 100.0% for primaquine, from 97.5 – 100.7% for impurity B, from 97.4 – 101.1% for impurity C, and from 96.3 – 102.2% for impurity D (cf. **Table 2**). Repeatability precision ranged from 98.5 – 100.7, 93.1 – 99.2, 96.0 – 99.1, and 96.1 – 99.7% for primaquine, impurity B, impurity C, and impurity D, respectively. The % RSD ranged from 0.4 – 13.0% for all substances (cf. **Table 3**). All values were within the acceptable limits of 85 – 115% and 0 – 15% RSD [18, 19]. Thus, the method was found to be precise and accurate.

Table 2: Recovery study results for primaquine and impurities B, C, and D.

Substance name	Target level (%)	Added amount (µg/mL; *n* = 21)	Recovered amount (µg/mL) (% recovery ± SD) (RSD) ; *n* = 21)
Primaquine	0.020	0.101	0.099 (98.4% ± 6.790) (6.9%)
	0.030	0.152	0.151 (99.6% ± 8.446) (8.5%)
	0.040	0.202	0.200 (99.1% ± 4.633) (4.7%)
	0.050	0.253	0.250 (98.8% ± 2.128) (2.2%)
	0.060	0.303	0.298 (98.4% ± 2.000) (2.0%)
	0.070	0.354	0.354 (100.0% ± 5.547) (5.5%)
Impurity B	0.030	0.153	0.149 (97.6% ± 6.165) (6.3%)
	0.050	0.229	0.230(100.3% ± 2.250) (2.2%)
	0.060	0.305	0.305 (100.7% ± 2.50) (2.5%)
	0.080	0.382	0.382 (100.0% ± 0.954) (1.0%)
	0.090	0.458	0.452 (98.7% ± 0.357) (0.4%)
	0.100	0.534	0.521 (97.5% ± 6.31) (6.5%)
Impurity C	0.020	0.076	0.074 (97.4% ± 0.500) (0.5%)
	0.030	0.151	0.152 (100.9% ± 2.000) (2.0%)
	0.050	0.227	0.229 (101.1% ± 0.500) (0.5%)
	0.060	0.302	0.300 (99.3% ± 1.500) (1.5%)
	0.080	0.378	0.380 (100.7% ± 0.154) (0.2%)
	0.090	0.453	0.450 (99.3% ± 0.400) (0.4%)
Impurity D	0.008	0.0382	0.0368 (96.3% ± 2.111) (2.2%)
	0.015	0.0764	0.0781(102.2% ± 1.321) (1.3%)
	0.023	0.4446	0.11463(100.03% ± 1.253) (1.3%)
	0.030	0.1528	0.1525 (99.8% ± 1.210) (1.2%)
	0.040	0.1910	0.1930 (101.05% ± 1.090) (1.1%)
	0.050	0.2292	0.2261 (98.7% ± 0.666) (0.7%)

Table 3: Repeatability study results for primaquine and impurities B, C, and D.

Substance name	Target level (%)	Retention time (min) ±SD (RSD) (*n* = 12)	Added amount (µg/mL; *n* = 12)	Recovered amount (µg/mL; ±SD (RSD) (*n* = 12)
Primaquine	0.020	4.7 ± 0.050 (1.1%)	0.101	0.102 (100.7% ± 7.426) (7.4%)
	0.040	4.8 ± 0.096 (2.0%)	0.202	0.200 (98.8% ± 4.000) (4.0%)
	0.060	4.8 ± 0.096 (2.0%)	0.304	0.300 (98.5% ± 1.638) (1.7%)
Impurity B	0.030	6.2 ± 0.096 (1.6%)	0.153	0.146 (95.4% ± 6.700) (6.6%)
	0.060	6.2 ± 0.141 (2.3%)	0.305	0.284 (93.1% ± 12.1) (13.0%)
	0.090	6.2 ± 0.100 (1.6%)	0.458	0.455 (99.2% ± 0.968) (1.0%)
Impurity C	0.020	5.5 ± 0.096 (1.7%)	0.076	0.073 (96.0% ± 3.500) (3.6%)
	0.060	5.5 ± 0.115 (2.1%)	0.304	0.301 (99.1% ± 1.271) (1.3%)
	0.090	5.5 ± 0.096 (1.7%)	0.456	0.451 (99.1% ± 0.369) (0.4%)
Impurity D	0.008	21.3 ± 0.818 (3.8%)	0.038	0.037 (96.1% ± 3.400) (8.1%)
	0.030	21.5 ± 0.926 (4.3%)	0.153	0.153(99.7% ± 0.844) (0.8%)
	0.050	21.2 ± 0.757 (3.6%)	0.229	0.227 (98.9% ± 0.800) (0.8%)

3.2.4 Robustness

The method is robust against slight changes of the percentage of methanol in the mobile phase (66.5 – 70%), the column temperature (25 – 35 ºC), the flow rate (0.65 – 0.85 mL/min), and the pH of the mobile phase (2.5 – 3.0) in terms of resolution among closely eluting substances like impurity A and primaquine as well as impurity C and primaquine (cf. **Figure 4**). However, the method was not robust utilizing more than 73.5% methanol in the mobile phase as it resulted in the coelution between impurity A and primaquine as well as a significant decrease of the resolution between impurity B and C from 2.2 at 70% methanol to 1.6 at 73.5%.

As expected temperature changes affected the retention times (impurity D R_t = 21.0 min at 25 ºC vs. 17.7 min at 35 ºC) which is in line with findings reported by Wolcott et. al [20]. The back pressure was monitored throughout the whole method development, optimization, and validation process, and ranged from 138 – 150 bar which is within an acceptable range considering the respective column dimensions.

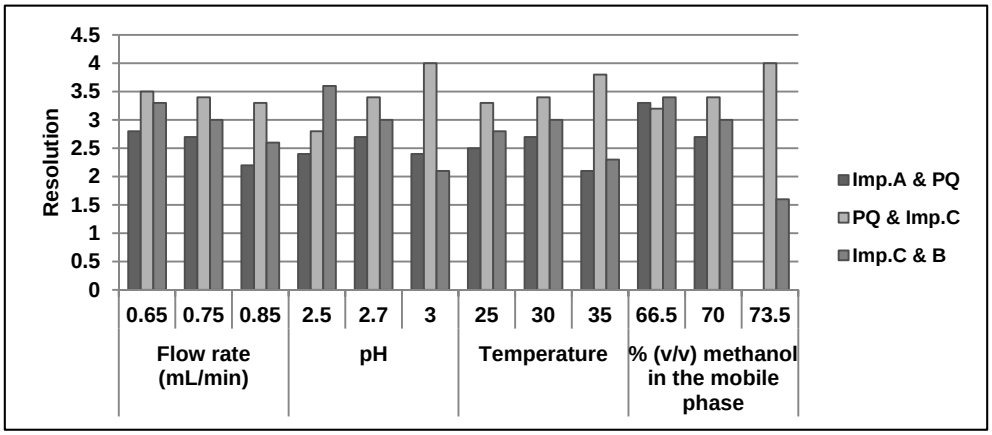

Figure 4: Robustness studies of the optimized method for primaquine and its three closely eluting impurities.

3.2.5 Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD of impurities B, C, and D ranged from 0.024 – 0.004 µg/mL, while the LOQ was between 0.038 and 0.15 µg/mL (cf. **Table 4**). The percentages of impurities peak area from the main peak area ranged from 0.003 to 0.013% for LOD and from 0.01 to 0.03% for LOQ. Impurity A was not commercially available thus not investigated.

Table 4: Limit of Detection (LOD) and Limit of Quantification (LOQ).

Substance	LOD (µg/mL)	% concentration from API (500 µg/mL)	% from API Peak	LOQ (µg/mL)	% concentration from API (500 µg/mL)	% API peak area
Primaquine	0.02	0.004	0.013	0.10	0.02	0.03
Impurity A			Not investigated			
Impurity B	0.012	0.0024	0.003	0.15	0.03	0.013
Impurity C	0.012	0.0024	0.007	0.07	0.014	0.023
Impurity D	0.012	0.0024	0.005	0.038	0.008	0.012

3.2.6 Related substances/synthetic impurities testing

Two batches (1 and 2) of primaquine were investigated for the presence of the aforementioned synthetic impurities using the optimized method (**Figure 5**) as well as the protocol for primaquine bulk drugs provided by the manufacturer (**Figure 6**). The sample solutions were prepared in duplicate and injected twice for each method. The Relative Response Factor (RRF) was also evaluated for each impurity and was within an acceptable limit of 0.8 – 1.2 for all impurities except for impurities B and D. Their correction factors of 3.48 and 0.71, respectively, were established and incorporated in the content determination (cf. **Table 1**).

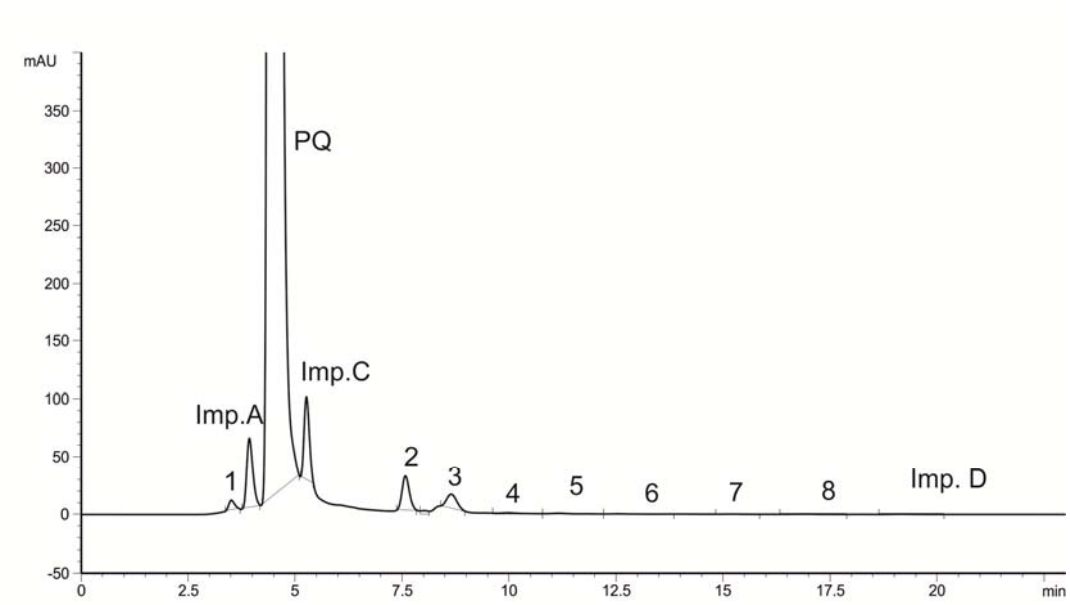

Figure 5: Chromatogram obtained from real sample of primaquine at 0.50 mg/mL using the optimized method (signals 1-8 are unknown).

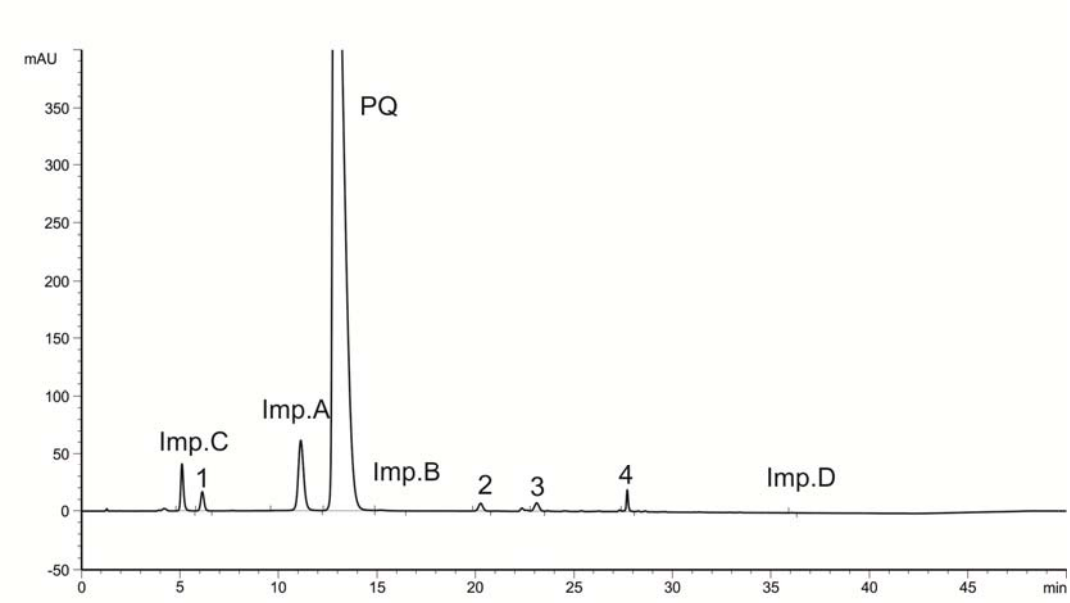

Figure 6: Chromatogram obtained from real sample of primaquine at 0.50 mg/mL using the current manufacturer method (signals 2-4 are unknown).

When using the manufacturer protocol the two batches did not comply with the requirements for the unspecified impurities, impurity A, impurity C, and the amount of total impurities. When analyzed with the optimized method, batch 1 did not comply with requirements for the unspecified impurities, impurity C, and total impurities, while batch 2 did not comply with requirements for impurity C as well as the unspecified impurities (**Table 5**).

Table 5: Comparison of impurity contents obtained from test solutions of the two batches tested using the optimized (A) and the current manufacturer method (B).

Impurity	Retention time (min)		Batch 1 [% content] (n=2)		Batch 2 [% content] (n=2)		Acceptable limits [% content]
	Method		Method		Method		
	A	B	A	B	A	B	
Unspecified	-	-	1.5	> 0.1	0.8	> 0.1	0.10
Primaquine	4.7	14.6	-	-	-	-	-
Imp.A	4.1	11.9	0.4	4.4	0.5	3.5	2
Imp.B	6.1	16.5	0.0	0.1	0.0	0.1	0.15
Imp.C	5.5	5.1	0.2	0.2	0.5	0.3	0.15
Imp.D	21.0	36.6	0.02	0.03	0.1	0.1	0.15
Total	-	-	2.1	6.8	1.7	5.8	2.0

When compared, statistically by *t*-test (Paired Two Sample for Means) at a level of 0.05% there is no significant difference between results obtained by using the manufacturer as well as the optimized method. Furthermore, results from these two methods were in total agreement in the case of the content of impurity C in batch 1 and that of impurity D for batch 2. However, the determined percentage content of impurity A in both batches was almost 10 times lower when the optimized method was applied (i.e., 0.4% for batch No.1 and 0.5 % for batch No.2) compared to 4.4% and 3.5%, respectively, using the manufacturer method. Furthermore, impurity B was not detected using the optimized method, nevertheless with the manufacturer method it was within the specified limits (< 0.1%).

The inconsistence observed could be ascribed to a difference in polarity for the investigated substances in which primaquine and impurities A, B, and C are polar while impurity D is relatively hydrophobic. Therefore, the optimized method condition is suitable for the detection and separation of all four impurities, but it suggests that impurity A is not fully resolved from primaquine. This is not favoured as the isomer might have undesired pharmacological activities. The investigation revealed that to get similar results, i.e. a content of 3 – 4% for impurity A, the amount of methanol in the mobile phase should not be more than 50%. However, at such a percentage of methanol, impurity D does not elute at all. It was also observed that the percentage content of impurity A decreases drastically from 4.4 and 3.5% to 0.4 or 0.5%, respectively, when the percentage of methanol in the mobile phase is higher than 50%. This was particularly the case at 66.5 – 70% methanol which on the other hand ensured a total elution of impurity D. The application of column B and C was not

helpful in improving the determination as in both cases it resulted in a very broad peak for impurity D. The deviation could also be ascribed to the use of different solvents: a mixture of acetonitrile, tetrahydrofuran, trifluoroacetic acid, and water was used in the manufacturer method, whereas a mixture of buffer and methanol was used for the optimized method. It is well known that acetonitrile and tetrahydrofuran are of higher resolving strength compared to methanol [6, 13, 21, 22]. Measurements at other wavelengths, i.e. λ = 215, 230, 254, and 261 nm do not affect the detection and quantitative determination of the affected impurities.

4. Conclusion

The newly developed protocol is very simple but still accurate, precise, and robust against slight changes of the most relevant chromatographic parameters affecting separation. The method offers significant advantages compared to compendial monographs (from the Ph. Eur., the B. P., the USP, and the Ph. Int., because it can be used for the simultaneous determination of the primaquine content and for impurity profiling, therefore being considered a suitable alternative. The new protocol offers a short analysis time (25 min) compared to the manufacturer's method (50 min) and can also be used for assay testing of primaquine in finished dosage forms.

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The authors declare no conflict of interest.

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3.6 Development and Validation of Simplified High-Performance Thin-Layer Chromatographic Methods for the Content Determination of Artesunate and Amodiaquine in Tablet Formulations

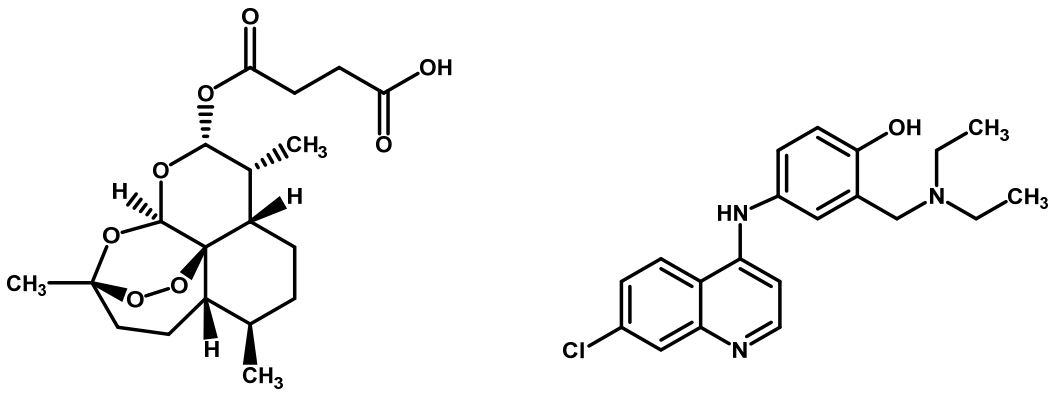
Abstract

Simple, cost-effective, precise, accurate, and rapid planar chromatographic methods were developed and validated for the separation and determination of amodiaquine and artesunate in tablet formulations. Both compounds were determined using high-performance thin-layer chromatography plates and a mobile phase composed of toluene, acetonitrile, methanol, ammonium acetate, and triethylamine in the ratio 10 : 5 : 3 : 1 : 0.5 (% v/v/v/v/v). Amodiaquine was evaluated densitometrically at a detection wavelength of λ = 345 nm, whereas artesunate was determined fluorimetrically at λ = 503 nm. The method was linear in the concentration ranges of 18.5 – 56 µg/mL for artesunate and 50 – 150 µg/mL for amodiaquine, respectively.

Keywords: artesunate; amodiaquine; counterfeit and substandard medicines; planar chromatography; densitometry; quality control; malaria

1. Introduction

Amodiaquine (4-[(7-chloro-4-quinolinyl)amino]-α-diethylmaino)methylphenol), together with chloroquine, belongs to the 4-aminoquinoline group of antimalarial drugs which are effective in the erythrocyte stage of *plasmodial* infection [1]. Artesunate is the hemisuccinate ester of dihydroartemisinin and co-formulated with amodiaquine (see **Figure 1**) or mefloquine in order to prevent the development of resistances and to potentiate the schizonticidal effect [2, 3].



Artesunate **Amodiaquine**

Figure 1: Chemical structural formulae of artesunate and amodiaquine.

These combinations are of clinical relevance particularly in the treatment of acute malaria and show a particular efficacy against chloroquine-resistant strains of *P. falciparum* [4, 5].

The separation techniques used for artesunate and amodiaquine include thin layer chromatography (TLC) [6, 7], high-performance chromatography (HPLC) [8-10], capillary electrophoresis [11], spectrophotometric methods [12, 13], and high-performance thin layer chromatography (HPTLC) [14, 15]. The International Pharmacopoeia describes separate monographs for the determination of artesunate and amodiaquine [16] while the United States Pharmacopoeia describes a monograph for amodiaquine only [17]. To date, there are no compendial monographs available for the simultaneous determination of dual combinations of artesunate and amodiaquine. Similarly, there are only three reported HPTLC methods for the simultaneous determination of these two substances; two involved double development of the precoated silica gel GF_{254} plates with two different mobile phases [6, 7] while the third applied a single development using one mobile phase [15]. Nevertheless, in all three methods the detection and quantitative determination of both substances were carried out after heating the plates at 150 ºC [15] and 180 ºC [7], respectively.

To provide users with a more simple alternative method to the HPTLC protocols described above a simple, precise, and accurate HPTLC method which can be easily applied in resource-constrained countries was developed. The method was developed and validated

for the content determination of proprietary medicines containing a fixed dose combination of 270 mg of amodiaquine and 100 mg of artesunate, utilizing pre-coated HPTLC silica plates and solvents such as acetonitrile, methanol, toluene, ammonium acetate, and triethylamine.

2. Experimental

2.1 Materials, chemicals, and equipment

Pre-coated HPTLC silica plates (60 $F_{254,}$ glass, 10 x 10 cm) and pre-coated TLC silica plates (60 F_{254}, aluminium, 5 x 10 cm) were used (all Merck, Darmstadt, Germany). A CAMAG HPTLC system coupled with a CAMAG ''Linomat'' semi-automatic applicator, a CAMAG developing tank, a CAMAG TLC scanner III, WinCATS-software (version 1.4.3) as data integrator, and a Hamilton syringe of 100 µL capacity for sample application (all CAMAG, Muttenz, Switzerland) was applied. Other instruments included an Ultrasonic bath from Wagtech International LTD (Berkshire, United Kingdom), and analytical balances from OHAUS Corporation (OHAUS, Pine Brook, NJ, USA) as well as from Mettler-Toledo (Greifensee, Switzerland). Toluene, triethylamine, and ammonium acetate were procured from Loba Chemie Pvt. Ltd (Mumbai, India), acetonitrile and methanol from Scharlau S.L. (Sentmenat, Spain), sulphuric acid from BDH Laboratory supplies (Poole, England), and purified water was generated by a Water Still, Aquatron A4000D from Barloworld Scientific LTD (Staffordshire, United Kingdom).

Artesunate and amodiaquine hydrochloride reference standards were obtained from the WHO Collaborating Centre for Chemical Reference Substances (Stockholm, Sweden) while tablets containing artesunate/amodiaquine (B. No. CYZ183012, Ipca Laboratories Ltd, India) were collected from the local market in Tanzania.

2.2 Solutions

2.2.1 Ammonium acetate buffer solution

A portion of 10.0 g of ammonium acetate was dissolved in 100.0 mL of distilled water.

2.2.2 Mobile phase

Volumes of 10 mL of toluene, 5 mL of acetonitrile, 3 mL of methanol, 1 mL of ammonium acetate buffer, and 0.5 mL of triethylamine were mixed. The developing chamber was saturated for 25 min.

2.2.3 Plate development

Volumes of 5.0 µL of each of the blank, standard, and sample solution were applied at a distance of 1.5 cm from either side or bottom margins of the plate. A minimum of three solutions was applied at each run (i.e. each one for the blank, standard, and sample solution). After development, the plate was dried at room temperature for at least 10 min. Amodiaquine was densitometrically evaluated at λ = 345 nm. Since artesunate does not have a chromophore it required derivatization using a mixture of methanol and sulphuric acid (19:1, % v/v) as specified in the GPHF monograph [18] to render it detectable at λ = 503 nm. This was achieved by spraying the plate after amodiaquine detection, heated at 75 ºC for 5 min for drying, followed by fluorescent densitometric measurement of the colored spots at λ = 503 nm.

2.2.4 Artesunate and amodiaquine stock standard solution

Portions of 3.7 mg of artesunate and 10.0 mg of amodiaquine were dissolved in 5 mL of methanol, sonicated for 10 min, and diluted to 10.0 mL with methanol, obtaining a stock standard solution containing 0.37 mg/mL of artesunate and 1.0 mg/mL of amodiaquine.

2.2.5 Sample solution

For each product, 20 tablets were weighed and ground to fine powder. An amount equivalent to approximately 3.7 mg of artesunate and 10.0 mg of amodiaquine was suspended in 5 mL of methanol and sonicated for 10 min. The suspension was diluted to 10.0 mL with methanol and filtered. Then 1.0 mL of the filtrate was subsequently diluted to 10.0 mL with the same solvent to make a final solution containing 0.037 mg/mL of artesunate and 0.1 mg/mL of amodiaquine.

2.4 Method development

Based on published methods [7, 14, 15, 18] the following pool of solvent combinations was investigated: toluene, methanol, and acetone in the ratio 5 : 5 : 15 (% v/v/v); ethylacetate and methanol in the ratio 25 : 5 (% v/v); toluene, methanol, and acetone 10 : 5 : 5 (% v/v/v); toluene, methanol, acetone 10 : 4 : 5 (% v/v/v); toluene and ethylacetate in the ratio 1 : 1 (% v/v), and toluene, ethylacetate, and acetone 10 : 5 : 5 (% v/v/v).

2.4.1 Method validation

The methods were validated for linearity, precision, specificity, and accuracy as per ICH guidelines [19].

2.4.2 Linearity of the calibration line

A standard stock solution containing 370 µg/mL of artesunate and 1000 µg/mL of amodiaquine was prepared in methanol and diluted using the same solvent to obtain five calibration solutions at levels of 18.0, 30.0, 37.0, 44.0, and 55.0 µg/mL for artesunate and 50.0, 80.0, 100.0, 120.0, and 150.0 µg/mL for amodiaquine. The solutions were applied to the plates and the analytical procedure was repeated on three consecutive days. The linearity was evaluated visually from plotted calibration data, and statistically by performing an F-test for the Lack-of-Fit (LOF).

2.4.3 Specificity

The specificity of the method was investigated by preparing the sample and standard solutions at a nominal concentration of 37.0 µg/ml for artesunate and of 100.0 µg/ml for amodiaquine and applied simultaneously with the mobile phase and the solvent on the HPTLC plate. The specificity of the method was assessed by comparing the densitogram and the R_f values obtained from standard solutions against those of sample solutions to ensure that none of them coeluted with the API or with each other.

2.4.3 Precision

Repeatability and intermediate precision were investigated by preparing six sample solutions at the nominal concentration of 37.0 µg/mL of artesunate and 100.0 µg/mL of amodiaquine.

Intermediate precision was performed by another analyst conducting analysis on three consecutive days [20].

2.4.4 Accuracy

Accuracy was studied by standard addition of a known amount of the two APIs to tablet sample solutions in triplicate at concentration levels of 80, 100, and 120% of the assay concentration and evaluating the accuracy of the analyte at each level [20].

3. Results and Discussion

3.1 Method Development

The objective of the study was to develop a simple HPTLC method for the simultaneous content determination of amodiaquine and artesunate in tablets.

The monograph from the GPHF manuals as well as published methods for the determination of artesunate and amodiaquine [7, 14, 15, 18] were used as a reference. Based on these published methods and the polarity of solvents, the following pool of solvent combinations was investigated: toluene, methanol, and acetone in the ratio 5 : 5 : 15 (% v/v/v); ethyl acetate and methanol in the ratio 25 : 5 (% v/v); toluene, methanol, and acetone 10 : 5 : 5 (% v/v/v); and toluene, methanol, and acetone 10 : 4 : 5 (% v/v/v). These solvent mixtures resulted in the reasonable separation of the two substances, however, tailing (due to the basic nature of amodiaquine) and considerably low R_f values (≤ 0.2 in the case of artesunate) occurred. Using a mixture of toluene and ethyl acetate in the ratio 1:1 (% v/v), the spot of the applied standard solution containing both substances did not move from the start position which can be ascribed to a lower elution strength of this solvent mixture. The addition of 1.0 mL of ammonium acetate solution to the mobile phase reduced the tailing of the amodiaquine spot and improved the resolution of the two compounds as well as peak height and area. Finally, using a mixture of toluene, acetonitrile, methanol, ammonium acetate solution, and triethylamine in the ratio 10 : 5 : 3 : 1 : 0.5 (% v/v/v/v/v) significantly improved the sharpness of the peaks, increased the R_f values, the peak heights and peak areas, and abolished tailing. Utilizing triethylamine instead of ammonia considerably improved the peak sharpness. Amodiaquine was densitometrically evaluated at λ = 345 nm,

while for artesunate a derivatization procedure had to be applied due to a lacking chromophore. Thus, a mixture of methanol and sulphuric acid (19:1, % v/v) was used as a spraying reagent as specified in the GPHF monograph [18] to render it detectable at λ = 503 nm (**Figure 2**)**,** heated at 75 ºC for 5 min for drying, followed by fluorescent densitometric measurement of the colored spots at λ = 503 nm (**Figure 3**). These detection wavelengths were obtained through scanning of the plates spotted with standard solutions of amodiaquine and the artesunate derivative within the range of λ = 200 – 700 nm.

The poor precision, as well as the low recovery of artesunate, could be attributed to incomplete derivatization because a simple glass sprayer for transfer of reagent and hot plate for heating were used. The use of such devices does not ensure the homogenous distribution of reagent over the layer and the spray is not fine enough. Furthermore, heating is critical for completion of the derivatization procedure at the desired temperature. The use of an inappropriate heating device may not ensure heating at the required temperature and therefore be responsible incomplete reaction.

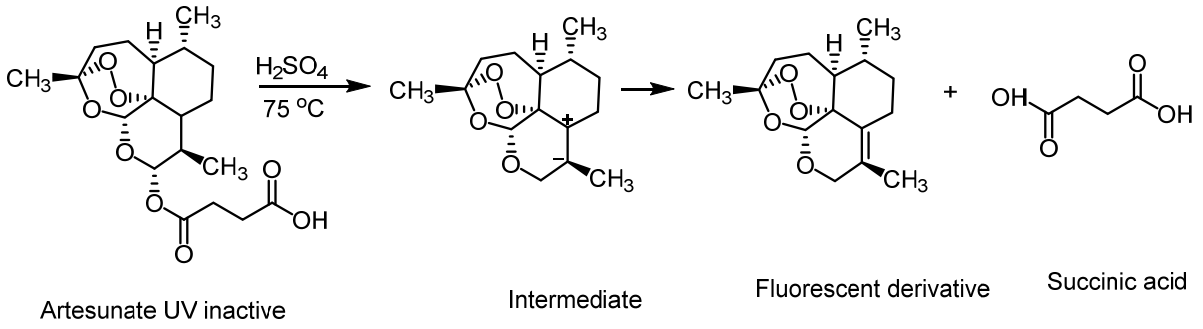


Figure 2: Reaction of artesunate with a mixture of methanol and sulphuric acid (19:1) to form a fluorescent derivative.

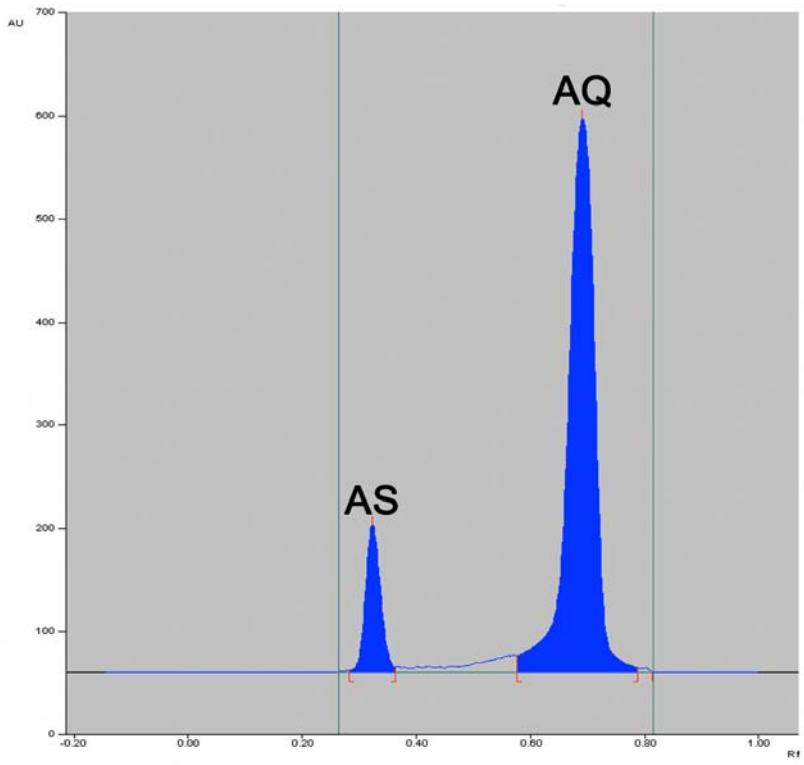


Figure 3: Densitogram showing the separation of artesunate (AS) and amodiaquine (AQ) using the optimized assay method.

3.2 Method validation

3.2.1 Specificity

The densitogram of the sample solution as well as the standard solution showed well separated signals of amodiaquine and artesunate, and none co-eluted with each other which suggests a specific method.

3.2.2 Linearity

The method was linear in the concentration ranges of 18.5 – 56.0 µg/mL and 50.0 – 150.0 µg/mL for artesunate and amodiaquine, respectively, with a regression coefficient of 0.996 for amodiaquine and 0.976 for artesunate (**Table 1**). Even though, the derivatization suffers from some disadvantages, the linearity is surprisingly good. The lack – of – fit test result for calibration data was F_{calc} = 0.227 for artesunate, and 0.0108 for amodiaquine. All values were below the critical value, F_{tab} (α = 0.05; df_1 = 3, df_2 = 15) = 3.490 indicating an adequate linear relationship between the spot areas and the concentrations for each compound.

Table 1: Linearity study results for amodiaquine and artesunate.

Level (%)	Amount took from stock solution (mL)	Final solution (mL)	Artesunate concentration (µg/mL)	Amodiaquine (µg/mL)
50	0.5	10.0	18.5	50.0
80	0.8	10.0	29.6	80.0
100	1.0	10.0	37.0	100.0
120	1.2	10.0	44.4	120.0
150	1.5	10.0	55.5	150.0
	Amodiaquine		Artesunate	
	Polynomial regression	Linear regression	Polynomial regression	Linear regression
Level (%)	50-150	50-150	50-150	50-150
x^2-coefficient	-3521.0	-	-6507.0	-
x-coefficient	18234.0	11181.0	9280.0	4471.0
y-intercept	6596.0	9703.0	396.9	1178.0
r^2	0.996	0.987	0.986	0.960
rsd	0.2	0.5	1.8	2.0

3.2.3 Precision

The repeatability was 1.1% for amodiaquine and 5.0% for artesunate. Intermediate precision of amodiaquine was 1.4% and 15.2% rsd for artesunate (**Table 2**). The rsd for the repeatability was ≤ 2% for amodiaquine and 5.0% for artesunate. The observed higher rsd for artesunate could also be attributed to incomplete derivatization as already discussed under 3.1.

Table 2: Repeatability and intermediate precision study results for artesunate and amodiaquine tablets solution.

Repeatability			
Parameter	**Artesunate**	**Amodiaquine**	
Level (%)	100	100	
Mean (peak area)	3032.010	22111.650	
sd	151.790	232.440	
% rsd	5.0	1.1	
n	6	6	
Intermediate			
Amodiaquine			
Parameter	**Analyst 1**	**Analyst 2**	**Average**
Level (%)	100	100	100
Mean(peak area)	22203.230	22112.000	22157.440
sd	373.560	232.440	300.460
%rsd	1.7	1.1	1.4
n	6	6	12
Artesunate			
Parameter	**Analyst 1**	**Analyst 2**	**Average**
Level (%)	100	100	100
Mean(peak area)	2299.600	3032.010	2665.800
sd	118.840	151.790	403.970
%rsd	5.2	5.0	15.2
n	6	6	12

3.2.4 Accuracy

The overall mean recovery ranged from 90 - 110% and from 98 - 102% for artesunate and amodiaquine, respectively (**Table 3**)**.** Recoveries were within the acceptable limit except for artesunate at both 80% and 120% levels.

Table 3: Recovery studies for artesunate and amodiaquine from spiked tablet solution.

	Amodiaquine			Artesunate		
Concentration levels	80%	100%	120%	80%	100%	120%
Mean	99.0	101.4	106.1	108.5	101.2	106.4
sd	1.26	1.57	1.74	6.62	12.44	3.95
% rsd	1.3	1.6	1.7	6.1	12.3	3.7
n	3	3	3	3	3	3

3.2.5 Laboratory cross validation

The % rsd for both protocols, i.e. the originator, who developed and validated the method, and the collaborator, being a 2nd laboratory that verified the method, was below 2.0% for amodiaquine, while in the case of artesunate it was 5.0% and 6.3% for both originator and collaborator, respectively. The interlaboratory variation was 1.3% and 5.7% for amodiaquine and artesunate, respectively **(Table 4)**, suggesting that the method is precise and robust. The higher variations observed for artesunate could be ascribed to the poor reproducibility of the derivatization process [21, 22].

Table 4: Comparison of repeatability precision study results obtained from two laboratories.

Parameter	Amodiaquine			Artesunate		
	Originator	Collaborator	Interlab	Originator	Collaborator	Interlab
Level (%)	100	100	100	100	100	100
Mean (peak area)	22111.65	22707.32	22409.48	3032.01	3263.64	3147.83
sd	232.45	369.24	300.85	151.79	206.86	179.32
%rsd	1.1	1.6	1.3	5.0	6.3	5.7
n	6	6	12	6	6	12

4. Conclusion

Despite the general challenges associated with the derivatization of artesunate, the optimized HPTLC method is simple, precise, accurate, and robust for the determination of amodiaquine. The derivatization problem for artesunate can be improved by employing an accurate HPTLC spraying as well as a heating device which will ensure that there is fine spray, homogeneous distribution of reagent, and that the plate is evenly heated to the

specified temperature. This will ensure that the derivatization process is completed and thus improve precision, accuracy, as well as linearity of artesunate. This study has also demonstrated that HPTLC methods may not be used as alternative to HPLC methods due to their limited precision, accuracy, and robustness. However, HPTLC can be applied for screening purposes.

Nevertheless, the optimized method provides an alternative to other reported methods which require double development of the plate using two different mobile phases. This is additional work to the analyst and increases the analysis costs in terms of solvent consumption [6, 7]. The new method also showed better performance when compared to reported methods in terms of precision and linearity in which linear regression coefficients ranged from 0.986 – 0.960 for artesunate and 0.987 – 0.996 for amodiaquine. Precision was < 2% for amodiaquine and 5.0% for artesunate. Adewuyi et. al reported intraday and interday precision ranging from 10.7 – 25.8% and 10.7 – 20.2%, respectively, for artesunate and 8.3 – 37.3 and 8.3 – 19.7%, respectively, for amodiaquine. The correlation coefficients ranged from 0.87 – 0.895 for artesunate and 0.859 – 0.945 for amodiaquine [6]. Accuracy was not investigated. In case of Nguyen et. al, the method was not fully validated and reported results were based on one solution prepared at 100% for both sample and standard thus a comparison is not warratted [7]. However, the reported results suggested a good method performance too.

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4. Final Discussion

This work aimed at developing methods for antimalarial drugs which are easy to be performed and suitable for determining the content of the API, evaluating the impurity profile, and detection of a different API than labeled. The methods should also be implemented using non-automated, modular instruments equipped with standard reversed phase columns and a UV detector using a cheap mobile phase (i.e., aqueous buffers and/or methanol). As an achievement of this study, three precise, accurate, and robust isocratic HPLC methods and two HPTLC methods for sulfadoxine, sulfalene, pyrimethamine, primaquine, artesunate as well as amodiaquine have been developed and validated as summarized in **Table 1**.

Table 1: Overview of Developed Methods

API HPLC	Column	Buffer type and molarity	% of buffer	MeOH (%v/v)	pH	Flow (mL/min)	λ (nm)	Runtime
Sulfalene, sulfadoxine, pyrimethamine	A,B	6.805g/L (0.05M) KH_2PO_4	60	40	2.6	1.5	215	15
Pyrimethamine	A	6.805g/L (0.05M) KH_2PO_4	40	60	2.6	1.2	215	30
Primaquine	C	3.13g/L (0.05M) NH_4HCO_2	30	70	2.7	0.75	265	25

HPTLC Method	Stationary phase	Mobile phase (%v/v/v)	λ (nm)
Sulfalene, sulfadoxine, pyrimethamine	Pre-coated TLC silica plates ($60F_{254}$, aluminium, 20 x 10 cm)	Toluene, ethyl acetate, and methanol (50 : 28.5 : 21.5)	254
Amodiaquine and artesunate	Pre-coated HPTLC silica plates ($60F_{254}$, glass, 20 x 10 cm) HPTLC plates	Toluene, acetonitrile, methanol, ammonium acetate, and triethylamine	345-amodiaquine & fluorescent artesunate derivative = 503

Column A: Eurospher-II C_{18}H column (250 x 4.6 mm, 5 µm), Column B: Eurospher-II C_{18}A column (250 x 4.6 mm, 5 µm), and Column C: XTerra RP_{18} column (250 x 4.6 mm, 5 µm)

4.1 Sulfadoxine, sulfalene and pyrimethamine content determination as well as sulfadoxine and sulfalene impurities determination method

The aim was to simultaneously determine the three APIs, the detection and separation of sulfonamides which are structurally related to these APIs, as well as their respective degradation products and synthetic impurities. This was achieved by using two standard C_{18} columns, phosphate buffer, and methanol, applying an isocratic elution mode. In addition,

the method is also capable of separating a potentially genotoxic API, dapsone, which might be present in the respective finished products as a fraudulent substitute [1]. This method offers several advantages compared to reported compendial monographs. Even though the fixed dose combination of sulfalene and pyrimethamine is still being widely used for over a decade now, the Ph. Int. monograph is limited to content determination and impurity profiling of sulfadoxine and pyrimethamine only. The monograph also makes use of triethylamine (an organic modifier) [2, 3] which can irreversibly adsorb to the stationary phase. Furthermore, expensive acetonitrile is among the solvents used unlike methanol utilized in the optimized method because it is relatively inexpensive and readily available in good quality in resource-constraint countries. The runtime for impurity profiling is more than 60 min compared to 15 min for the method developed here. The USP monograph is also limited to content determination of sulfadoxine and pyrimethamine only, utilizing a sophisticated phenyl HPLC column and acetonitrile [4]. The B. P. only described a monograph for pyrimethamine while Ph. Eur. prescribed content determination of sulfadoxine and pyrimethamine utilizing titration as well as TLC for impurities profiling [5, 6]. The Ph. Int. monograph can also only detect sulfamethoxazole in the presence of sulfadoxine compared to nine structurally related sulfonamides with the new method. Generally, compendial monographs are only capable of detecting drugs having a lower content as well as the presence or absence of the declared API. However, it has to be said that the pharmacopoeial methods are intended to evaluate the quality of one API only. Statistical evaluation of content determination of these three APIs indicated that there is no significant difference among the sets of results obtained using the new method and the Ph. Int. monograph for sulfadoxine and pyrimethamine, which suggest that the method described here can be used as an alternative to these compendial monographs. However, slightly lower contents for pyrimethamine were obtained using the optimized method in one of the analyzed proprietary product (Ekelfin) which can be attributed to different tablet excipients and a matrix effect as this was not observed in the case of other *sulfalene/pyrimethamine* generic brands.

4.2 Pyrimethamine content determination and impurity profiling method

The objective was to separate four impurities of pyrimethamine (starting materials and intermediate products of synthesis), as well as toluene (solvent) from the main peak. This was achieved by employing a commercially available, inexpensive reversed phase C_{18} column, phosphate buffer, and methanol applying an isocratic elution mode accomplishing the analysis within 30 min.

The current compendial monographs for pyrimethamine in the Ph. Eur. and the B. P. describe the determination of the related substances utilizing thin layer chromatography (TLC) which is less sensitive compared to HPLC. The USP as well as the Ph. Int. do not have provisions for related substances but describe assay testing by liquid chromatography or a non-aqueous titration, respectively [2-4]. Additionally, the USP and B. P. describe monographs for the content determination of pyrimethamine in tablet formulations by using a UV/Vis spectroscopic method and a wavelength of λ = 272 and 273 nm, respectively [3, 4].

4.3 Primaquine content determination and impurity profiling method

The aim of this investigation was to separate and quantify the API and the four synthetic impurities of primaquine; an isomer of primaquine, starting material, and intermediate products. This was achieved by employing a standard reverse phase (C_{18}) column, an ammonium formate buffer solution, and methanol, applying an isocratic elution mode, resulting in a total runtime of 25 min. This method has several advantages compared to the currently reported compendial monograph. For example, the current protocol for primaquine in the B. P. [6], describes the determination of primaquine and only one impurity by utilizing normal phase chromatography which need specialized accessories (column, suitable valves) where for highly hydrophobic solvents e.g. hexane) compared to reversed phase where standard C_{18} columns and inexpensive and readily available methanol and buffers are used. The USP monograph, apart from utilizing acetonitrile and tetrahydrofuran as well as trifluoroacetic acid, also applies a C_8 column which might not be readily available and less retentive compared to standard C_{18} columns. However, it also lacks a detailed description of specified impurities except for impurity A (8-(4-aminopentylamino)-6-methoxyquinoline) and

secaquine [4]. The Ph. Int. depicted related substance testing by TLC [3] which is less sensitive compared to HPLC. Despite of these advantages of the newly developed method, the percentage content of impurity A in the tested batches was almost 10 times less compared to those obtained using manufacturer method (i.e., 0.4% for batch No.1 and 0.5 % for batch No.2 using the optimized method compared to 4.4% and 3.5%, respectively). This observation needs further investigation in order to improve the method and might be attributed to the difference in polarity for the investigated substances in which primaquine, and its impurities A, B, and C are polar while impurity D is non-polar (hydrophobic). The chromatographic conditions described by the optimized method ensure that all five substances are detected and separated, but they are not suitable for the total separation of impurity A from primaquine, even though the resolution between them is ≥ 2. For the quantitative determination of impurity A the methanol content in the mobile phase must be not higher than 50%, resulting in a percentage content above 4.0% which is in-line with manufacturer method observations. At this condition, impurity D remains stacked in the column due to its hydrophobicity and does not elute at all until when utilizing methanol in the range between 66.5 to 70% during which the content decreases to less than 1%.

Nevertheless, the observed difference could also be ascribed to the use of different solvents in which a mixture of acetonitrile, tetrahydrofuran, trifluoroacetic acid, and water was used for the manufacturer method compared to the mixture of buffer and methanol utilized in the optimized method. It is well known that acetonitrile and tetrahydrofuran are of higher resolving strength than methanol [7]. It is anticipated that further investigation will address the observed gap.

4.4 Sulfalene, sulfadoxine and pyrimethamine HPTLC content determination method

The aim was to develop a method for the simultaneous determination of sulfadoxine, sulfalene as well as pyrimethamine in their pharmaceutical formulation. This was achieved by applying standard TLC plates and inexpensive, readily available and safe solvents like ethyl acetate, methanol, and toluene as mobile phase and carrying out densitometric evaluation by scanning the developed plate at a wavelength of λ = 254 nm. Application of

densitometric determination of pharmaceutical substances in current compendial monographs is limited to herbal medicines analysis in the Ph. Eur. [8] while TLC is prescribed in identification as well as impurities limit test [4, 6, 8, 9].

Statistical evaluation of sets of results obtained by implementation of the method on proprietary products containing sulfalene/pyrimethamine and sulfadoxine/pyrimethamine previously analyzed using the Ph. Int. sulfadoxine/pyrimethamine monograph revealed no significant differences which suggests that the method can be used as an alternative to compendial monographs involving HPLC.

Nevertheless, the method is not suitable for the simultaneous determination of all three APIs. However, this is not a problem because sulfalene and sulfadoxine are not formulated together, thus they can easily be separated because they are available in separate products. The method is also devoid of capacity to detect other APIs than the declared one thus it is not suitable to detect API exchanges for counterfeiting.

4.5 Artesunate and amodiaquine content determination HPTLC method

The aim of the method was to simultaneously separate and quantify the two APIs in their respective pharmaceutical formulations. This was achieved using pre-coated HPTLC silica plates and non-hazardous solvents such as acetonitrile, methanol, toluene, ammonium acetate, and triethylamine. To date, there are no compendial monographs available for the simultaneous determination of dual artesunate/amodiaquine formulations. The Ph. Int. describes separate monographs for the determination of artesunate and amodiaquine [9] while the USP describes an amodiaquine monograph only [4]. Similarly, there are only three reported HPTLC methods for the determination of these two substances, in which two involved double development of the described plates, i.e. a different mobile phase for each substance has to be used [10, 11] while the third applied a single development only [12]. Nevertheless, in all three methods, the detection and quantitative determination of both substances was carried out after heating the plate at 150 ºC [12] and 180 ºC [11] using precoated silica gel GF_{254}. This optimized method provides users with an alternative to

HPTLC methods involving heating the plates before detection and quantitative determination.

However, the optimized method has a limitation when it comes to the quantitative determination of artesunate which requires derivatization prior to its detection and determination. The use of a derivatizing agent introduces bias and much variation in the results obtained. Nevertheless, the performance of the method is not too bad.

5. Conclusion

The outcome of this entire study has demonstrated that it is possible to develop cheap, simple, precise, accurate, and robust HPLC methods for antimalarial and antibiotic medicines while employing inexpensive, readily available, and safe solvents such as methanol and simple buffers. It was also concluded that these methods have a sensitivity, precision, as well as accuracy comparable to that offered by compendial methods. The possibility of buffer precipitation within the system or column which could lead to abrasion of pump seals or total damage of the column [13, 14] can be easily avoided by ensuring that all buffers are prepared within the concentration range of 10 – 50 mM. This is the recommended range for reverse phase chromatographic methods. In addition, the consumption of chemicals is reduced by this strategy.

The fact that these methods are operated in an isocratic mode ensures that they can be implemented even in laboratories with simple single pump HPLC systems only. Isocratic elution modes are more reproducible and robust compared to gradient methods; even though the number of compounds that can be resolved might be lower [14]. However, the developed methods are very economic and easy to operate.

The HPTLC methods can also be used as an alternative to the pharmacopoeial monographs, particularly in countries where densitometric equipment is already available. In case where an HPLC instrument is already in place, they can be used for screening tests or for quick quality evaluations, followed by a comprehensive quality analysis.

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6. Summary

Although the prevalence of substandard and counterfeit pharmaceutical products is a global problem, it is more critical in resource-constrained countries. The national medicines regulatory authorities (MNRA) in these countries have limited resources to cater for regular quality surveillance programmes aimed at ensuring that medicines in circulation are of acceptable quality. Among the reasons explained to hinder the implementation of these strategies is that compendial monographs are too complicated and require expensive infrastructures in terms of environment, equipment and consumables. In this study it was therefore aimed at developing simple, precise, and robust HPLC and HPTLC methods utilizing inexpensive, readily available chemicals (methanol and simple buffers) that can determine the APIs, other API than declared one, and which are capable of impurity profiling. As an outcome of this study, three isocratic and robust HPLC and two HPTLC methods for sulfadoxine, sulfalene, pyrimethamine, primaquine, artesunate, as well as amodiaquine have been developed and validated. All HPLC methods are operated using an isocratic elution mode which means they can be implemented even with a single pump HPLC system and standard C_{18} columns. The densitometric sulfadoxine/sulfalene and pyrimethamine method utilizes standard TLC plates as well as inexpensive, readily available and safe chemicals (toluene, methanol, and ethyl acetate), while that for artesunate and amodiaquine requires HPTLC plates as well as triethylamine and acetonitrile due to challenges associated with the analysis of amodiaquine and poorly the detectable artesunate. These HPTLC methods can

be implemented as alternative to those requiring HPLC equipment e.g. in countries that already have acquired densitometer equipment. It is understood that HPTLC methods are less sensitive, precise and accurate when compared to HPLC methods, but this hindrance can easily be addressed by sending representative samples to third party quality control laboratories where the analytical results are verified using compendial HPLC methods on a regular basis.

It is therefore anticipated that the implementation of these methods will not only address the problem of limited resources required for medicines quality control but also increase the number of monitored targeted antimalarial products as well as the number of resource-constrained countries participating in quality monitoring campaigns. Moreover, the experiences and skills acquired within this work will be applied to other API groups, e. g. antibiotics, afterwards.

7. Zusammenfassung

Trotz der weltweiten Verbreitung gefälschter Arzneimittel und solcher, die nicht die deklarierte Menge an Wirkstoff enthalten, sind vor allem Entwicklungs- und Schwellenländer von dieser Problematik betroffen. Die Arzneimittelüberwachungs- bzw. Zulassungsbehörden dieser Länder verfügen nur über eingeschränkte Möglichkeiten, die Arzneimittelqualität regelmäßig zu überwachen und somit sicherzustellen, dass die im Markt befindlichen Medikamente eine gute Qualität aufweisen. Einer der Gründe hierfür ist unter anderem, dass die in Arzneibüchern beschriebenen Methoden oftmals sehr komplex sind und eine umfassende Laborausstattung, spezielle Geräte oder teure Chemikalien benötigen. In dieser Arbeit wurden einfache, genaue und robuste flüssigchromatographische Methoden entwickelt, die lediglich günstige, überall verfügbare Chemikalien (z. B. Methanol oder einfache Puffersalze) benötigen und mit denen der Gehalt des deklarierten Arzneistoffes, Arzneistoffverwechslungen sowie das Verunreinigungsprofil bestimmt werden kann. Es konnten drei isokratische, robuste flüssigchromatographische sowie zwei

dünnschichtchromatographische Methoden zur Bestimmung von Sulfadoxin, Sulfalen, Pyrimethamin, Primaquin, Artesunat sowie Amodiaquin entwickelt und validiert werden. Alle flüssigchromatographischen Methoden arbeiten isokratisch, folglich können sie auch mit sehr einfachen HPLC-Geräten mit beispielsweise nur einem Pumpenkopf genutzt werden. Zudem werden nur einfache, kommerziell erhältliche C_{18}-Säulen benötigt. Die densitometrischen Methoden für Sulfadoxin/Sulfalen sowie Pyrimethamin benötigen standardisierte Dünnschichtchromatographie-Platten sowie günstige, überall verfügbare und wenig toxische Chemikalien wie beispielsweise Toluol, Methanol oder Ethylacetat. Für die Methode zur Bestimmung von Artesunat und Amodiaquin werden Hochleistungsdünnschichtchromatographie-Platten und Triethylamin sowie Acetonitril benötigt. Dieser Umstand ist der Tatsache geschuldet, dass Amodiaquin und Artesunat sich anderweitig nur ungenügend trennen ließen. Die dünnschichtchromatographischen Protokolle können als Alternative zur HPLC eingesetzt werden, beispielsweise überall dort, wo bereits die entsprechenden Gerätschaften vorhanden sind. Natürlich weisen dünnschichtchromatographische Methoden im Vergleich zur Flüssigchromatographie eine geringere Sensitivität, Präzision und Richtigkeit auf, dies kann jedoch dadurch umgangen werden, die entsprechenden Methoden nur zum *Screening* zu verwenden und die zu analysierenden Proben anderweitig, z. B. in externen Laboratorien, detailliert zu untersuchen. Dort können beispielsweise Methoden aus gängigen Arzneibüchern verwendet werden. Durch die Implementierung der neu entwickelten Methoden kann zum einen das Problem schlecht verfügbarer Chemikalien umgangen werden und gleichzeitig die Anzahl an untersuchten Arzneimitteln erhöht werden. Dies ist ein wichtiger Beitrag zur Qualitätskontrolle in Ländern mit eingeschränkten Infrastrukturen.

8. Appendix

a) A list of publications and documentation of authorship

1 **Routine quality control of medicines in developing countries: Analytical challenges, regulatory infrastructures and the prevalence of counterfeit medicines in Tanzania**

Hoellein L, Kaale EA, Mwalwisi YH, Schulze MH, Holzgrabe U:.

TrAC 2016;76:7660–70

2 **Development of a simple, rapid, and robust liquid chromatographic method for the simultaneous determination of sulfalene, sulfadoxine, and pyrimethamine in tablets**

Mwalwisi YH, Hoellein L, Kaale E, Holzgrabe U

J Pharm Biomed Anal 2016; 129:558-570

3 **Development of a Simple, Rapid, and Robust Isocratic Liquid Chromatographic Method for the Determination of Pyrimethamine and its Synthetic Impurities in Bulk Drugs and Pharmaceutical Formulations**

Mwalwisi YH, Hoellein L, Kaale E, Holzgrabe U, Malar J

4 **Development and Validation of Simplified High-Performance Thin-Layer Chromatography Densitometric methods for the Determination of Sulfalene, Sulfadoxine, and Pyrimethamine in Tablet Formulation**

Mwalwisi YH, Hoellein L, Shewiyo DH, Holzgrabe U, Kaale E, Journal of Tropical Medicines and international Health

5 **Development and Validation of a Simple, and Robust Isocratic Liquid Chromatographic Method for the Determination of Primaquine and its Synthetic Impurities in Bulk Material and Pharmaceutical Formulations**

Mwalwisi YH, Hoellein L, Kaale E, Holzgrabe U, J Clin Pharm

6 **Development and Validation of Simplified High-Performance Thin-Layer Chromatography Densitometric methods for the Determination of Amodiaquine, and Artesunate in Tablet Formulation**

Omolo SC, Mwalwisi YH, Hoellein L, Shewiyo DH, Holzgrabe U, Kaale E, J Clin Pharm

9. A list of individual contribution for each author to the publication reprinted in this thesis

P1 Hoellein L, Kaale EA, Mwalwisi YH, Schulze MH, Holzgrabe U**,** Routine quality control of medicines in developing countries: Analytical challenges, regulatory infrastructures and the prevalence of counterfeit medicines in Tanzania. TrAC 2016;76:7660–70

Author	1	2	3	4	5
Review	X	X	X	X	X
Data analysis	X	X	X		X
Manuscript planning	X	X	X	X	X
Manuscript writing	X	X	X	X	X
Correction of manuscript	X	X	X	X	X

P2 Mwalwisi YH, Hoellein L, Kaale E, Holzgrabe U, Development of a simple, rapid, and robust liquid chromatographic method for the simultaneous determination of sulfalene, sulfadoxine, and pyrimethamine in tablets. J Pharm Biomed Anal 2016; 129:558-570

Author	1	2	3	4
Study design	X	X	X	X
Experimental work	X			
Data analysis	X			
Manuscript planning	X	X	X	X
Manuscript writing	X	X		X
Correction of manuscript	X	X	X	X
Supervision of Mwalwisi YH				X

P3 Mwalwisi YH, Hoellein L, Kaale E, Holzgrabe U, Development of a Simple, Rapid, and Robust Isocratic Liquid Chromatographic Method for the Determination of Pyrimethamine and its Synthetic Impurities in Bulk Drugs and Pharmaceutical Formulations. Malar J

Author	1	2	3	4
Study design	X	X	X	X
Experimental work	X			
Data analysis	X			
Manuscript planning	X			X
Manuscript writing	X	X	X	X
Correction of manuscript	X	X	X	X
Supervision of Mwalwisi YH				X

P4 Mwalwisi YH, Hoellein L, Kaale E, Holzgrabe U, Development and Validation of Simplified High-Performance Thin-Layer Chromatography Densitometric methods for the Determination of Sulfalene, Sulfadoxine, and Pyrimethamine in Tablet Formulation. Journal of Tropical Medicines and International Health

Author	1	2	3	4
Study design	X		X	X
Experimental work	X		X	
Data analysis	X	X	X	
Manuscript planning	X	X	X	X
Manuscript writing	X	X	X	X
Correction of manuscript	X	X	X	X
Supervision of Mwalwisi YH			X	X