DEVELOPMENT AND VALIDATION OF A LIQUID CHROMATOGRAPHIC METHOD FOR THE ANALYSIS OF AZITHROMYCIN

A thesis submitted in partial fulfillment of requirements for the award of the degree of Master of Pharmacy in Pharmaceutical Analysis of the University of Nairobi

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2013

DECLARATION

This research thesis is my original work and has not been presented elsewhere for award of any degree.

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DEDICATION

This research work is dedicated to my dear wife, Loice Wambui and my daughter, Ruth Moraa, for enduring my periodic absence from home while undertaking experimental work.

To my parents, Mr. Okaru Nyaturu and Mrs. Agnes Moraa Okaru and my siblings, Cosmas Nyaturu, Richard Aondo, Lydiah Nyaboke and Zachary Mwembi for their encouragement and support all my life.

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LIST OF SYMBOLS AND ABBREVIATIONS

CAN	Acetonitrile
AEA	Anhydroerythromycin A
AZA	Azathromycin
AZT	Azithromycin
AZTNO	Azithromycin N-Oxide
BP	British Pharmacopoeia
BWD	Box and Wilson Design
CPP	Critical Peak Pair
CRS	Chemical Reference Standard
CV	Coefficient of variation
°C	Centigrade (degrees)
DAZT	Decladinosylazithromycin
DRA	Drug Regulatory Authority
EAEN	Erythromycin A enol ether
EAIE	Erythromycin A imino ether
EAOX	Erythromycin A oxime
GC	Gas Chromatography
H	Hour(s)
HPLC	High Performance Liquid Chromatography
ICH	International Committee on Harmonization
IR	Infra-red
k'	Capacity factor

Kg	Kilogram
LC	Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantitation
M	Molar (concentration)
Mg	Milligram(s)
Min	Minute(s)
MoH	Ministry of Health
m.p.	Melting point
NDMAZT	N-demethylazithromycin
NQCL	National Quality Control Laboratory
pK_a	Acid dissociation constant
PsEAEN	Pseudoerythromycin A enol ether
PsEAHK	Pseudoerythromycin A hemiketal
R^2	Coefficient of determination
RP	Reverse Phase
RSD	Relative Standard Deviation
RT	Retention time
S/N	Signal to Noise (Ratio)
TBAOH	Tetrabutylammonium hydroxide
USP	United States Pharmacopoeia
UV	Ultra-violet
WRS	Working Reference Standard

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DEFINITION OF TERMS

Accuracy	Closeness of test results obtained by the method to the true value.
Capacity Factor	Measure of where the peak of interest is located with respect to the elution time of the non-retained components.
Linearity	Ability of method to give responses that are directly proportional to the concentration of the analyte within a given range.
Method Optimization	The systematic variation of chromatographic factors in order to obtain optimum or satisfactory conditions.
Precision	Degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of a homogenous sample.
Robustness	Measure of the capacity of the method to remain unaffected by small but deliberate variations in the method parameters.
Selectivity	Relative retention calculated for two adjacent peaks.
Sensitivity	Ability to detect and quantify small changes in the concentration of the analyte in the sample.
Specificity	Ability to assess unequivocally the analyte in the presence of components that may be expected to be present such as impurities, degrades or matrix components.
Stress Testing	Studies taken to elucidate the intrinsic stability of the active pharmaceutical ingredient under severe conditions.

ABSTRACT

Azithromycin is a semi-synthetic macrolide antibiotic that is active against a number of Gram-positive and Gram-negative bacteria. Azithromycin is listed in the World Health Organization and the Ministry of Health, Kenya essential drugs list. Therefore, the quality of azithromycin products in the market is important because of its role in management of atypical infections. Liquid chromatography is the method of choice for the analysis of azithromycin in bulk samples and its formulations. However, the current published liquid chromatographic methods employ extreme conditions of pH and temperature and also expensive columns for the analysis of azithromycin.

In this study, a simple, isocratic, rapid, sensitive and robust reverse phase HPLC method was developed for the analysis of azithromycin in tablets, suspensions and raw materials. The effect of chromatographic factors including use of inorganic mobile phase buffer, pH, ion-pairing agent, organic modifier and temperature were investigated during development. Waters XTerra® C18 column having the dimensions of 250×4.6 mm and 5 µm particle size with mobile phase containing a mixture of acetonitrile-0.1M KH_2PO_4 pH 6.5-0.1M tetrabutylammonium hydroxide-water (25:15:1:59 % v/v/v/v) was employed to achieve separation of azithromycin and its related substances. The pH of the buffer and ion pairing agent was brought to 6.5 using an equimolar solution of 0.1M K_2HPO_4. The mobile phase was delivered at a flow rate of 1 ml/min and the components were monitored at 215 nm while the column temperature was maintained at 43 °C. The retention time of azithromycin was found to be about 8 min.

The proposed method was validated using ICH guidelines for the parameters of linearity, recovery, precision, sensitivity and robustness. The method was found to be linear in the range 50-150 percent with the coefficient of determination (r^2) being 0.997. The LOD and LOQ were found to be 20 εg and 78 εg respectively. The method was found to exhibit good accuracy with the percent recovery of azithromycin being 100.7 %. The coefficient of variation for the repeatability and intermediate precision were within the limits prescribed by ICH.

The robustness ranges for the critical factors of pH, temperature and acetonitrile were investigated with the aid of Statgraphics Centurion XVI. The robustness range for pH was 6-7 pH units, 22-28% v/v for acetonitrile and 41-45 °C for temperature. At 95% confidence interval, p=0.05, acetonitrile had the biggest impact on the chromatographic parameters of the critical peak pair of azithromycin and erythromycin A imino ether.

The degradation of azithromycin in 0.0005% v/v H_2O_2 was found to be second order (r^2=0.994) with a half life of 13 min. In 0.1M H_3PO_4 and 0.05M H_3PO_4, 90% of azithromycin decayed within 10 min. In 0.025M H_3PO_4, the degradation was found to be first order with r^2 being 0.9907 with a half life of 57.8 h.

The developed method was successfully applied in the analysis of azithromycin raw material and twenty two commercial products obtained from retail pharmacies within the Central Business District of the City of Nairobi. Thirteen of these products were tablets while nine were suspensions of azithromycin. Five of the nine suspensions sampled were premixed suspensions

while the rest were dry powders for reconstitution. Three tablet brands did not meet the United States Pharmacopoeia (2012) specifications for assay (90.0-110.0% label claim) while three of the premixed azithromycin suspensions did not meet the USP (2012) specifications. Similarly, one of the four dry powders for reconstitution suspensions failed to comply with the assay specifications.

The developed method is simple, robust, specific, accurate and stability indicating. It can therefore be used by quality control laboratories in the routine analysis of azithromycin bulk samples and formulations as well as in stability studies. The method can also be applied in post market surveillance to monitor the quality of products in circulation and detect counterfeits.

CHAPTER ONE

GENERAL INTRODUCTION

1.1. The macrolide antibiotics

Macrolides antibiotics are a large group of antibiotics consisting of macrocyclic lactone rings to which amino and deoxy sugar moieties are attached. They are produced through fermentation as a complex mixture of closely related components which are extracted using water-immiscible solvents followed by reduction *in vacuo*. The extract is then separated and purified by chromatography to individual components. The semisynthetic erythromycins are obtained by partial chemical derivatization of erythromycin A [1].

Various strains of *Streptomyces* species are the source of a number of structurally related macrolides antibiotics collectively known as erythromycins. However, rosaramicin and mirosamycin are isolated from *Micromonospora* species. The semisynthetic derivatives of erythromycin A include roxithromycin, dirithromycin, clarithromycin, flurithromycin and azithromycin [1].

Erythromycin A is the most active of the macrolides from natural sources and it has been routinely used for the treatment of upper and lower respiratory tract infections as well as skin and soft tissue infections as it has activity against Gram-negative and Gram-positive microorganisms [2].

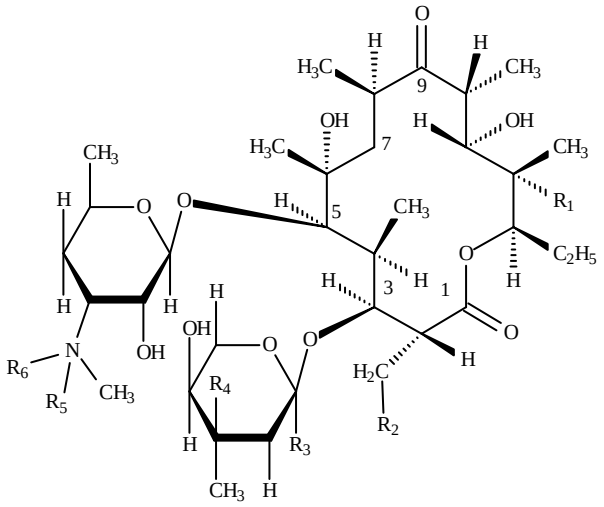
1.2 Classification of macrolides

The most commonly used macrolide antibiotics consist of a macrocyclic lactone ring containing 14, 15 or 16 atoms linked by glycosidic to amino sugars and deoxy sugars [3].

The clinically useful macrolide antibiotics can be conveniently classified into three groups based on the number of atoms in the lactone nucleus. These are 14, 15 and 16-membered macrolides. Erythromycins, oleandomycin, roxithromycin, dirithromycin, clarithromycin and flurithromycin, telithromycin are 14-membered whereas azithromycin is 15-membered. The 16-membered macrolides include josamycin, carbomycin A, rosaramicin, rokitamycin, kitasamycin, mirosamicin, spiramycin, troleandomycin, midecamicine acetate and tylosin. Spiramycin and tylosin are used almost exclusively in veterinary medicine [4].

Erythromycin A is a 14-membered macrolide with desosamine and cladinose as the amino and deoxy sugars respectively. The structures of erythromycin A-F and other related substances are shown in Figure 1.1. The amino sugar, desosamine, is linked via ß-glycosidic bonds while cladinose, a deoxy sugar, is linked via Ť-glycosidic bonds. The deoxy sugar is glycosidically linked at C3- and the amino sugar at C5-.

The 14 and 16 membered macrolides are obtained naturally from the micro-organisms while the 15 membered macrolides are synthesized form erythromycin A with ring expansion and are called ‘azalides’. Azithromycin is an azalide in clinical use.

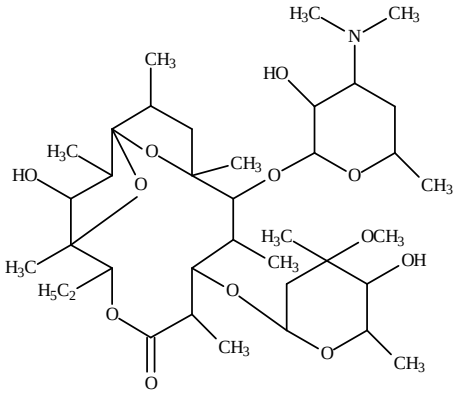


	R_1	R_2	R_3	R_4	R_5	R_6
Erythromycin A (EA)	OH	H	H	OCH_3	CH_3	-
Erythromycin B (EB)	H	H	H	OCH_3	CH_3	-
Erythromycin C (EC)	OH	H	H	OH	CH_3	-
Erythromycin D (ED)	H	H	H	OH	CH_3	-
Erythromycin E (EE)	OH	-O-		OCH_3	CH_3	-
Erythromycin F (EF)	OH	OH	H	OCH_3	CH_3	-
N-demethylerythromycin A	OH	H	H	OCH_3	-	-
Erythromycin A N-oxide (EANO)	OH	H	H	OCH_3	CH_3	O

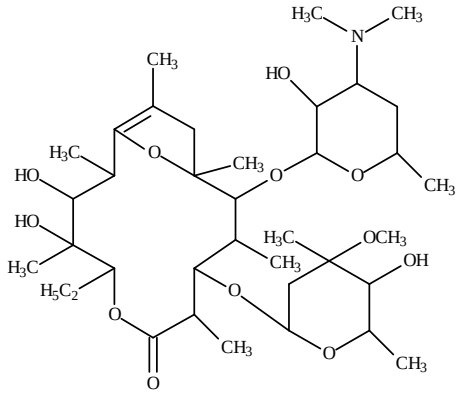
Figure 1.1: Chemical structures of erythromycins

Erythromycin A exhibits poor bioavailability and produces gastrointestinal side effects that arise from its degradation in acidic medium. Under the acidic conditions of the stomach, erythromycin

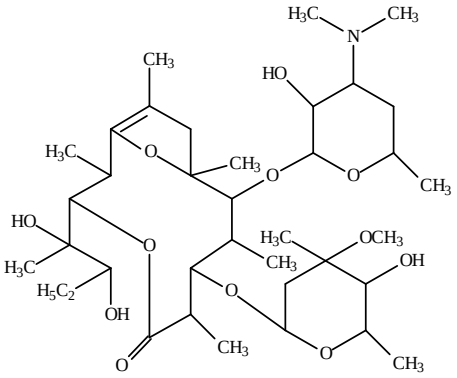
A quickly degrades into erythromycin A enol ether (EAEN) and anhydroerythromycin A (AEA) which are inactive as antibiotics and thus impart poor bioavailability to erythromycin. Erythromycin A enol ether has been shown to enhance the stimulation of intestinal peristalsis and it accounts for the gastrointestinal discomfort associated with ingestion of erythromycin A. Figure 1.2 shows the structures of some degradation products of EA. The pseudoerythromycins, PsEAEN and PsEAHK, are degradation products in basic environment [5].



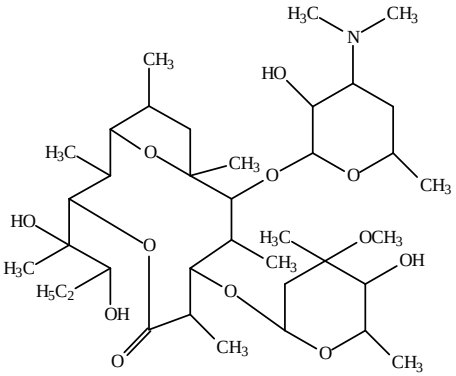
Anhydroerythromycin A (AEA)



Erythromycin A enol ether (EAEN)



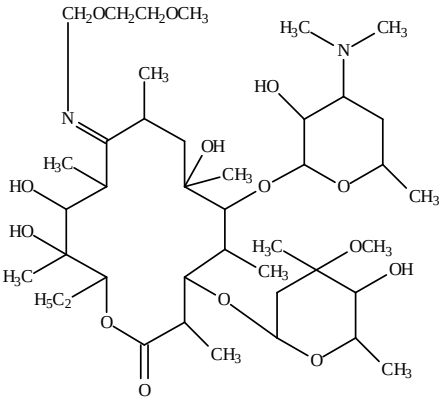
Pseudoerythromycin A enol ether (PsEAEN)



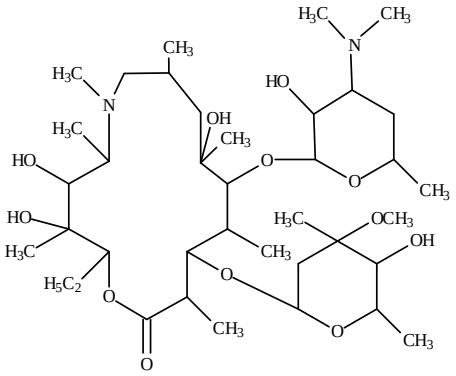
Pseudoerythromycin A hemiketal (PsEAHK)

Figure 1.2: Chemical structures of some of the degradation products of erythromycin A.

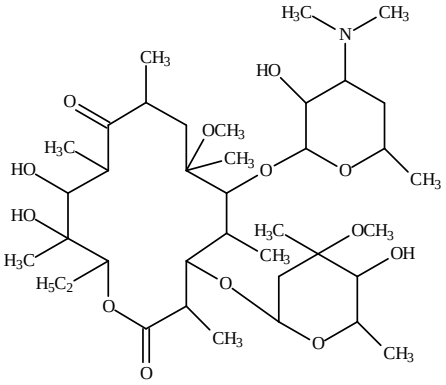
Intensive search for macrolides with improved acid stability but still maintaining good overall spectrum of activity has resulted in generation of several semi-synthetic derivatives of erythromycin A. The macrolides, clarithromycin, roxithromycin and azithromycin have enjoyed great clinical success due to their enhanced antibacterial activity, improved pharmacokinetic properties, expanded spectrum of activity and attenuated gastro-intestinal side effects as compared to erythromycin A. In addition, ketolides which lack the C3-cladinose have been found to be potent antibiotics with strong ribosomal binding ability. Telithromycin is an example of a ketolide in clinical use [2]. Figure 1.3 shows the chemical structures of some of the semisynthetic derivatives of erythromycin A.



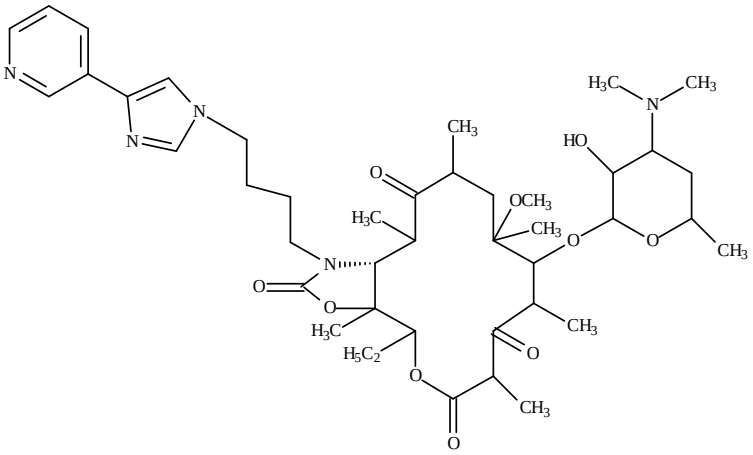
Roxithromycin



Azithromycin



Clarithromycin



Telithromycin

Figure 1.3: Chemical structures of some semisynthetic derivatives of erythromycin A

1.3 Mechanism of action of macrolides

The macrolide antibiotics exert their antibacterial activities by reversibly binding to the 50S component of the 70S ribosomal subunit in the bacterial cell. This interaction inhibits RNA-dependent protein synthesis by preventing transpeptidation and translocation reactions causing the premature detachment of incomplete peptide chains, resulting in subsequent cell death [6, 7]. Macrolides bind to domain V of the 23S ribosomal RNA (rRNA) [7]. The ketolides bind with a

10-to 100-fold higher affinity to the ribosome than erythromycin. Additionally, the ketolides, unlike other macrolides, have a greater affinity to bind to domain II of the 23S rRNA enabling it to maintain antimicrobial activity against bacterial strains that are macrolide resistant because of alterations in the domain V binding site [8]. This action is mainly bacteriostatic, but it can be bactericidal in high concentrations. Macrolides accumulate within leucocytes before they are transported to the site of infection [8].

It has been suggested that the higher ribosome binding affinity of AZT may account for its enhanced activity against Gram-negative microorganisms [9]. However, if the ribosome is derived from a resistant strain of bacteria with an *erm* gene that results in modification of the 50S ribosomal subunit, the inhibitory activity of AZT is partially lost [7, 10, 11].

1.4 Antibacterial activity of macrolides

All macrolide antibiotics display similar antibacterial properties and are active against Gram-positive and some Gram-negative bacteria. They are particularly active against *Mycoplasma, Haemophilus influenzae, Mycobacterium avium, Neisseria gonorrhoeae, Chlamydia* species and *Rickettsia*. They are therefore indicated for the management of atypical pneumonia caused by *Chylamydia pneumoniae* and *Mycoplasma pneumoniae*. They do not have activity against *enterococci* and methicillin-resistant *Staphylococcus aureus.* In particular, macrolide antibiotics constitute an important alternative for patients exhibiting penicillin sensitivity and allergy where they are used as second line drugs. Clarithromycin is used as a component of the multi-drug combination in the treatment of gastric ulceration due to *H. pylori* [6, 12].

Macrolide antibiotics except azithromycin have also been shown to have immunomodulatory effect of stimulating macrophages and also studies have shown that erythromycin, clarithromycin and azithromycin possess anti-inflammatory characteristics in patients with respiratory diseases who do not show evidence of bacterial infection [12].

1.5 Resistance

Macrolide resistance in *Streptococci* principally arises from either an alteration of the drug-binding site on the ribosome by methylation which is responsible for macrolide-lincosamide-streptogramin B (MLS) cross resistance [13]. The MLS resistance depends on the methylation of the ribosomal binding site by some micro-organisms such as *Staphylococcus aureus*. Resistance by methylation of an adenine residue in domain V of the 23S rRNA is mediated by the erythromycin ribosome methylase (erm) genes. Methylation prevents binding of the macrolides and ketolides to domain V and results in high-level macrolide resistance (MICs 64 mg/L). Active drug efflux mechanism also accounts for macrolide resistance and is mediated by the macrolide efflux (mef) genes that are speciýc for 14- and 15- membered macrolides [7]. Such macrolide resistance is usually low level with the minimum inhibitory concentrations (MICs) of 1-32 mg/L and *in vitro* susceptibility to ketolides, lincosamides and streptogramins is maintained [13].

Ketolides presumably maintain their antimicrobial activity by virtue of their ability to bind to an alternative site, domain II of the 23S rRNA [8]. Methylase may either be induced or constitutively expressed and resistance to erythromycin implies cross-resistance to clarithromycin and azithromycin [14]. Both clarithromycin and azithromycin can induce methylase production resulting in resistance. The 3-ketone substitution of telithromycin, however, does not induce methylase production [15].

Some of the factors that contribute to the development of azithromycin-resistant microbes include residues excreted into waste water in sewers [16] and low concentration that may persist in the body after completion of treatment [16-18].

Resistance to AZT is more prevalent in Gram-positive than in Gram-negative bacteria [7, 19, 20, 21]. In the case of Gram positive organisms with the type *erm* gene, bacterial resistance is thought to result from the induction or constitutive production of an enzyme, *erm* 23S-rRNA methyltransferase, which catalyses methylation of adenosine residues in the bacterial ribosome [7-11, 19]. Methylation of the adenosine moiety is responsible for the transfer of resistance to erythromycin and other 14-membered macrolide antibiotics [7]. Cross-resistance with erythromycin occurs since azithromycin is incapable of inhibiting methylated ribosomes especially in Gram-positive organisms [7]. *Streptococcus pneumoniae* has the *erm* B gene while *Staphylococcus aureus* has *erm* A and *erm* C gene [7]. Resistance to azithromycin is also thought to be associated with the presence of an energy dependent efflux pump mechanism that is present in some microbes and appears to be specific for 15-membered ring macrolide antibiotics [19, 20]. Examples of microorganisms that show energy dependent efflux resistance mechanisms include *Streptococcus pneumoniae* encoded with *mefE* gene [7, 21] and *Staphylococcus pyogenes* encoded with *mefA* gene [7]

1.6 Adverse effects of macrolides

Gastrointestinal intolerance is the primary adverse side effect of macrolides when administered to man. The gastrointestinal side effects are greatly reduced for semi-synthetic macrolides compared to erythromycin [22]. Semi-synthetic macrolides such as azithromycin, clarithromycin

and telithromycin are well tolerated. The most common adverse effects reported with azithromycin are diarrhea, nausea, abdominal pain and headache or dizziness. The gastrointestinal side effects arise from the degradation of macrolides in acidic conditions.

Laboratory abnormalities were infrequent and minor including transient increases in transaminases in 1.5% of patients and decrease in leukocyte count. Adverse events related to the intravenous infusion of azithromycin are pain at the injection site and local inflammation [23]. Similar adverse reactions are reported with clarithromycin. There is no difference in the spectrum and frequency of adverse reactions between the extended-release or immediate-release formulations of clarithromycin [24]. Gastrointestinal adverse events with the extended-release formulation, however, are less severe and result in fewer discontinuations of the medication.

1.7 Azithromycin

1.7.1 Description

Azithromycin (AZT) is a macrolide antibiotic that is active primarily against various aerobic Gram-positive and some Gram-negative microorganisms. Azithromycin is rapidly absorbed and is widely distributed to tissues and concentrated in cells. Peak plasma concentration is achieved within 2-3 h [21]. Azithromycin presents pharmacokinetic and pharmacodynamic parameters that allow for a simple once a day dosing regimen with minimal side effects.

Azithromycin products are formulated as tablets, capsules, oral suspensions and intravenous preparations.

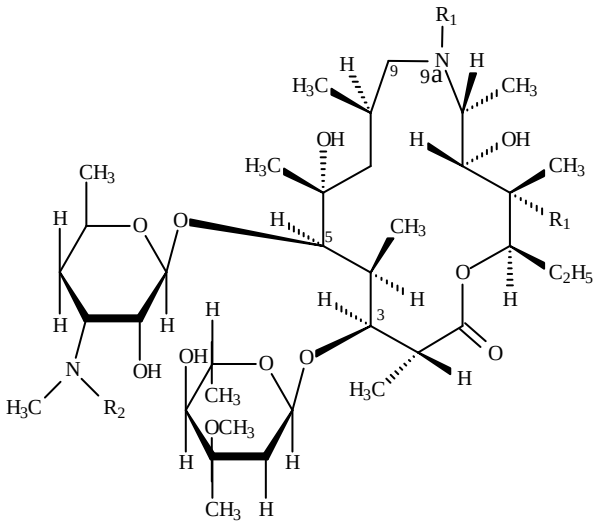
1.7.2 Chemistry

Azithromycin is a semisynthetic azalide macrolide derived from erythromycin A but composed of a 15-membered lactone ring. As in erythromycin, cladinose and desosamine sugar residues are attached at positions 3 and 5 respectively.

The IUPAC name of azithromycin is (2*R*,3*S*,4*S*,5*R*,8*R*,10*R*,11*R*,12*S*,13*S*,14*R*)-2-ethyl-3,4,10-trihydroxy-3,5,6,8,10,12,14-heptamethyl-15-oxo-11-{[3,4,6-trideoxy-3-(dimethylamino)-ß-D-*xylo*-hexopyranosyl]oxy}-1-oxa-6-azacyclopentadec-13-yl-2,6 dideoxy-3-*C*-methyl-3-*O*-methyl-Ŭ-L-*ribo*-hexopyranoside. Its molecular formula is $C_{38}H_{72}N_2O_{12}$, molecular weight is 748.98. The degree of hydration of AZT crystals is 1 or 2.

Azithromycin is formed by inserting a methyl-substituted nitrogen in place of the carbonyl group at the C9 position of the lactone ring. The resulting dibasic 15-membered ring macrolide derivative is referred to as anóóazalide.ôô

Figure 1.4 shows the chemical structures of azithromycin and some of its related substances. Azathromycin arises from synthesis while decladinosylazithromycin and N-demethylazithromycin are degradation products.



	R_1	R_2	Cladinose present
Azithromycin (AZT)	CH_3	CH_3	+
Azathromycin (Azaerythromycin) (AZA)	H	CH_3	+
N-demethylazithromycin (NDMAZT)	CH_3	H	+
Decladinosylazithromycin (DAZT)	CH_3	CH_3	-

Figure 1.4: Chemical structures of azithromycin and some of its related substances

Apart from a basic sugar with a tertiary ionizable amine group located at position C3N of desosamine, azithromycin has a second basic tertiary amine at position C9a that makes it a dibasic macrolide. The pKa_1 values range between 8.1-8.85 while that of pKa_2 fall between 8.8-9.45 [25-27]. The pKa_1 corresponds to the ionization of dimethylamino group on C3N while pKa_2 corresponds to the C9a amine.

1.7.3 Synthesis

Azithromycin is a product of the Beckmann rearrangement of the corresponding 9-(E)-erythromycin A oxime, a product arising from oximation of erythromycin A with hydroxylamine hydrochloride (Figure 1.5). Erythromycin A oxime undergoes Beckmann reactions to give either 9,11-iminoether or 3,11-iminoethers that upon catalytic hydrogenation yields azathromycin that further undergoes N-methylation to yield azithromycin. Since azithromycin is obtained from erythromycin A, impurities present may undergo the same synthetic modifications as erythromycin A and the azithromycin analogues of these impurities can be found in azithromycin bulk samples. In addition, degradation products of azithromycin as well as intermediates of the semi-synthetic pathway may be present.

From the synthetic scheme for azithromycin (Figure 1.5), erythromycin A oxime, erythromycin A imino ether, azathromycin are the related substances arising from synthesis. Decladinosylazithromycin and N-demethylazithromycin, arise from degradation. The determination of small amounts of degradation products in a vast excess of parent drug is difficult and this is confounded by the absence of a chromophore in those compounds [28].

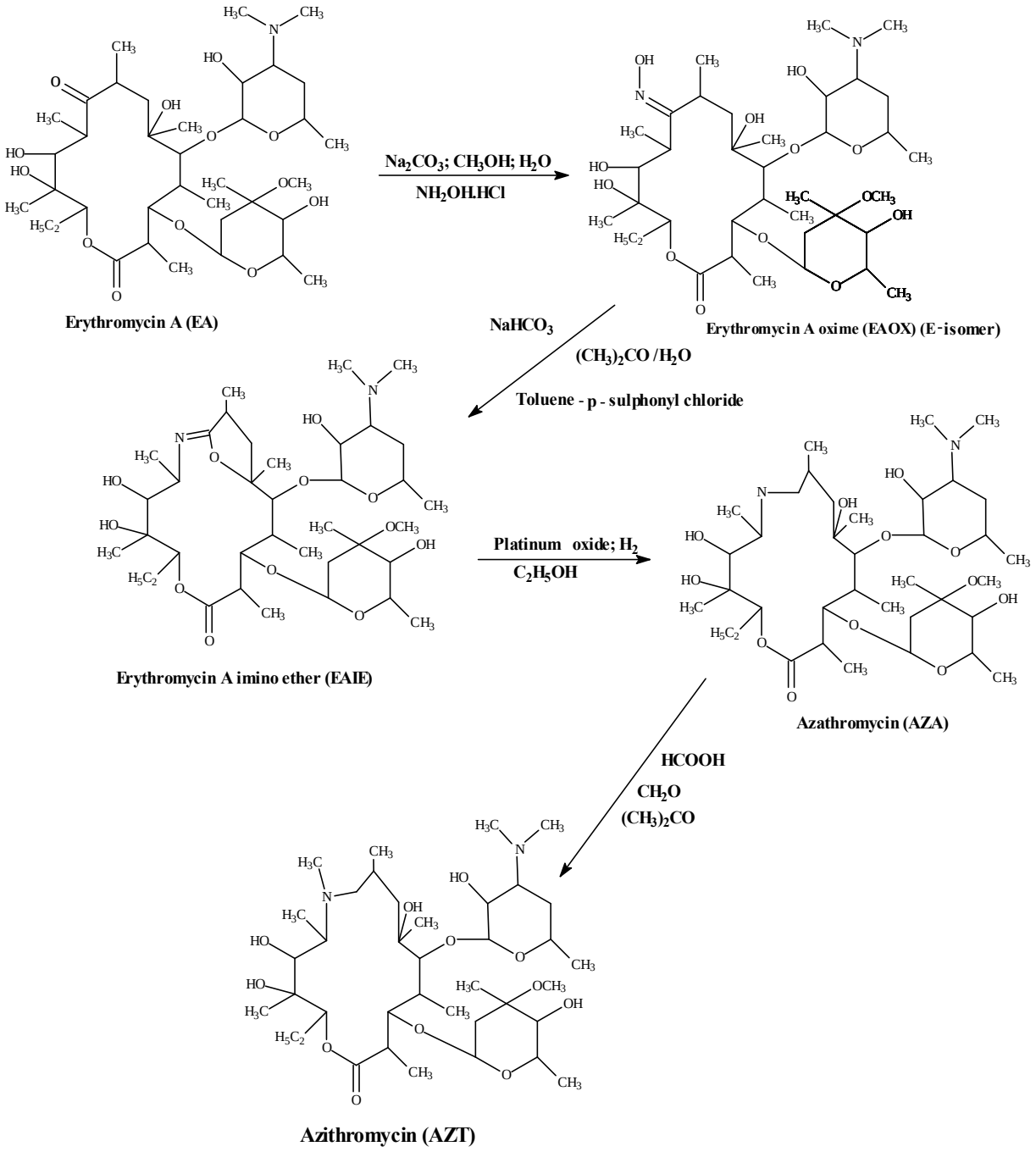


Figure 1.5: Synthetic scheme of azithromycin

1.7.4 Stereochemistry and structure activity relationship

The absolute stereochemistry azithromycin is shown in Figure 1.6. The configuration is R at C2, C5, C6, C8, C10, C11and C13 while it is S at C3, C4 and C12.

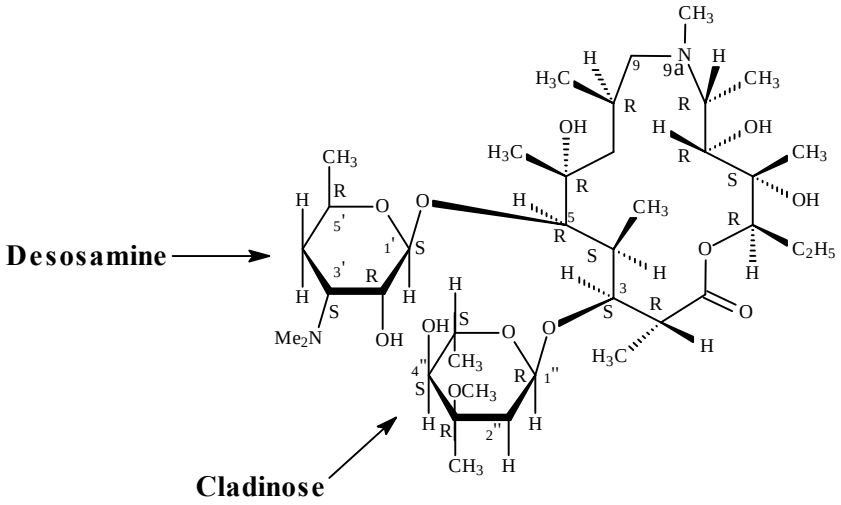


Figure 1.6: Stereochemistry of azithromycin

Modification of the structure of AZT affects its activity against bacterial pathogens, pharmacokinetic and pharmacodynamic properties. Cleavage of the macrolide ring structure has been reported to lead to loss of bactericidal activity [29]. The antibacterial activity of azalides decreases with an increase in the number of methyl groups of the macrocyclic backbone [29].

Removal of the tertiary amine group from the desosamine sugar at position C3N(Figure 1.6), greatly reduces the activities of macrolide agents including that of AZT [7]. Cladinose is an essential component for the antibacterial activity of these compounds [30]. In the ketolide family, the cladinose sugar at position C3 is replaced by a ketone group which does not trigger the expression to MLS resistance in microorganisms with *erm* determinants. Decladinosylation of erythromycin A reduces ribosomal binding ability and this is compensated by the addition of

the 11,12-carbamate group on ketolides. Ketolides have been shown to exhibit enhanced antibacterial potency when compared to erythromycin as a result of tighter binding to ribosomes [30].

Increased potency of macrolides has also been observed when the original basic macrolide ring of erythromycin A is modified by the addition of a methyl-substituted nitrogen at position C9. This modification has rendered AZT two to eight fold more active against *Haemophilius influenzae* than erythromycin [7, 10]. The addition of a methylamino group on the lactone ring also extended the range of antimicrobial activity of the macrolides against Gram negative organisms whilst retaining activity against Gram positive microorganisms. The replacement of the 9 carbonyl in the aglycone ring with a methyl substituted nitrogen, in addition to ring expansion to a 15 membered ring, also resulted in improved acid stability of AZT as compared to erythromycin [31, 32]. In addition to increased acid stability, formation of an anhydrohemiketal derivative, which has been associated with the gastrointestinal toxicity observed with erythromycin in acidic media is markedly reduced or even avoided [32]. These changes also increase the lipid solubility of the molecule, thus conferring unique pharmacokinetic characteristics and antimicrobial properties to AZT. The increased lipid solubility possibly accounts for the improved antibacterial activity as a result of higher tissue to serum levels [31].

1.7.5 Stability

In azithromycin, the insertion of a methyl-substituted nitrogen in place of the carbonyl group at the 9 position of the parent compound, erythromycin A, blocks the internal ketalization reaction that usually involves the C6-OH and the C9 keto to form the hemiketal [32, 33]. However, for azithromycin, acid hydrolysis of the ether bond to produce neutral cladinose sugar is the main

decomposition pathway [32]. Studies indicate a 300-fold increase in acid stability for azithromycin for decladinosylation, compared with erythromycin over the gastric pH range [32]. The two major degradation impurities of azithromycin are decladinosylazithromycin and N-demethylazithromycin.

The solubility of azithromycin in water improves when the pH of the solution is adjusted to 5.0 using citric acid, but this solution unstable and precipitates over time [34]. Addition of sodium salts such as sodium hydroxide stabilizes the solution but alters the pH to 7.0 [34].

The amine functionality of AZT located at position C3N in desosamine is amenable to oxidation and other degradation reactions if exposed to elevated temperatures and/or air during the development and manufacturing of AZT containing dosage forms [35, 36]. Michael *et al.* [35] performed degradation studies on AZT at an elevated temperature of 55 °C for a period of 3 months and used HPLC/MS-MS, NMR and UV-spectrophotometry to identify degradation products formed under these stress conditions. The presence of the degradation products may lead to deviation of the product from regulatory purity requirements during the shelf life [35].

The propensity of azithromycin to undergo oxidative degradation or decomposition under normal storage conditions may result in unacceptable levels of impurities being present in dosage forms [36, 37]. The susceptibility of AZT to degradation underscores the need to develop an optimal formulation and method of manufacture in which the stability of AZT is not compromised, resulting in the production of a high quality, stable, safe and effective dosage forms. A variety of strategies including the use of antioxidants and of gas impermeable laminated aluminium packaging [37, 37] have been shown to enhance the stability of AZT during manufacturing and

storage. This is important in the design of the new pharmaceutical dosage forms with respect to storage and packaging of the product with the aim of minimizing the presence of unacceptable levels of impurities at the time of administration.

1.7.6 Clinical pharmacology

1.7.6.1 Spectrum of activity

Azithromycin exerts its antimicrobial activity against a large number of Gram-positive and Gram-negative bacteria that are associated with respiratory, gastrointestinal, skin and sexually transmitted infections.

The drug is of major importance in the treatment of intracellular pathogens such as *Chlamydia pneumoniae, Chlamydia trachomatis, Mycloplasma pneumonia* and *Legionella pneumonia* [7], as well as enteric pathogens such as *Salmonella typhi, Shigella flexneri, Shigella sonnei* and *Shigella dysenteriae* [11]. Although slightly less potent than erythromycin against Gram-positive microorganisms, the drug demonstrates superior activity *in vitro* against a wide variety of Gram-negative bacteria, including *Haemophilus influenzae*, *Moraxella catarrhalis* and *Haemophilus parainfluenzae* [7, 11]. *In vitro* potency testing of AZT is sensitive to the pH of the bacterial growth medium and a reduction in pH will result in the ionization of AZT, which alters its ability to penetrate into bacterial cells with a subsequent increase of the minimum inhibitory concentration (MIC) of the antibiotic [38].

Azithromycin is highly effective in inhibiting clinically significant intracellular pathogens such as *Chlamydia trachomatis* and *Legionella* [7, 10]. The drug is also effective against *Mycoplasma*

species such as *Mycoplasma hominis*, that are resistant to both erythromycin and tetracycline antibiotics [7]. In addition, the drug has also shown good *in vitro* activity against a number of non-bacterial intracellular pathogens such as the protozoan organism, *Toxoplasma gondii* [7, 10, 39].

Azithromycin can be either bactericidal or bacteriostatic, depending on the concentration of the molecule *in vivo*. A number of *in vitro* studies have shown AZT to possess bactericidal activity. Prolonged tissue concentrations above the MIC at the site of infection for several days [39] suggest that the drug may possess bactericidal effects *in vivo*. Azithromycin is inactive against methicillin-resistant *Staphylococci* and most strains of *Enterococcus fecalis*, as well as Gram-positive strains that are known to be resistant to erythromycin [7, 10].

1.7.6.2 Clinical indications

Clinical indications of AZT include the treatment of respiratory infections such as acute *Streptococcus pyogenes* pharyngitis, tonsillitis, otitis media, sinusitis, community-acquired pneumonia, acute bronchitis, acute exacerbations of chronic bronchitis and acute bacterial exacerbations of chronic obstructive pulmonary disease [11]. Azithromycin can also be used for the treatment of skin and soft-tissue infections caused by susceptible pathogens [7, 10]. The treatment of urogenital and other sexually transmited infections caused by *Chlamydia trachomatis, Neisseria gonorrhoeae*, *Hemophilus ducreyi* and *Ureaplasma urealytecum* can be achieved by the use of a single one gram dose of AZT [7].

Azithromycin is the antibiotic of choice for the treatment of legionnairesô disease [7, 40] and can be used for the prevention of disseminated *Mycobacterium avium* complex (MAC) disease in

patients with advanced Human Immunodeficiency Virus (HIV) infections [41]. It can be used in combination with ethambutol, for the treatment of *Mycobacterium avium* intracellular complex infection in patients with advanced HIV infections and also for prophylaxis alone or in combination with rifabutin against MAC [41, 42]. In addition, AZT may also be used for the treatment of typhoid fever [7, 43], trachoma [7, 43], cryptosporidiosis and toxoplasmosis [7, 43, 44] as well as gastrointestinal infectious diseases caused by pathogens, such as those caused by the *Shigella* and *Salmonella* species [10, 39].

1.7.7 Pharmacokinetics

1.7.7.1 Absorption and distribution

The oral bioavailability of AZT following administration of a single 500 mg oral dose to healthy male volunteers in the fasted state is estimated to be 37% while the peak serum concentration is achieved approximately two hours after the dose [11, 21, 45, 46]. Food reduces the peak serum concentration by up to 50% and the AUC by 43% [11] for capsules. Oral bioavailability of the tablets and oral suspension are not affected by meals [46].

Following oral administration, azithromycin is rapidly distributed to the tissues such as tonsils and prostate reaching high and sustained concentrations of up to 10-100 times greater than those found in serum [45]. The drug also concentrates in leukocytes, monocytes, alveolar cells, polymophonuclear lymphocytes, fibroblasts and macrophages [20, 47, 48]. The presence of high concentrations of AZT in phagocytic cells allows for the release of the antibiotic at local sites of infection and inflammation. The high penetration of azithromycin into microorganisms may be responsible for its expanded spectrum of activity especially against intracellular infective

organisms [10, 49]. The transport of AZT into cells by both passive and active processes is probably due to its dibasic amphiphilic nature [50].

Azithromycin binds primarily to Ŭ- and ɓ- globulins, but not to serum albumin [11]. Protein binding of AZT also correlates to Ŭ$_1$-acid glycoprotein levels, thus patients with elevated levels of Ŭ$_1$-acid glycoprotein have an increase in AZT protein binding [11, 38, 39].

1.7.7.2 Metabolism and elimination

Small amounts of azithromycin are demethylated in the liver and excreted in bile as unchanged drug and inactive metabolites. The principal metabolic pathways for azithromycin include N-demethylation of the desosamine sugar to form N-desmethylazithromycin or at the 9a position of the macrolide ring to form 9a-N-desmethylazithromycin [7, 21, 50]. The 9a-N-desmethylazithromycin metabolite has been shown to be active against many strains of bacteria [51]. The remainder is excreted mainly as the parent substance unchanged in urine [48].

Azithromycin an also undergo O-demethylation of the C3[illegible]OCH_3 of cladinose, decladinosylation and hydroxylation of the desosamine or lactone ring. Hunter *et al.* [52] determined the profile of metabolites of AZT in plasma, bile, liver, lung, kidney and skin tissues using the Ball python snake as the animal model and established fifteen metabolites of AZT together with their structures and possible pathways of metabolism.

Elimination of azithromycin from the plasma occurs in a biphasic manner. A rapid decline in drug plasma concentration implies a rapid redistribution phase into tissues following

administration that is then followed by a second distribution component, which in turn is followed by biliary excretion, the major pathway of elimination [11, 50].

1.8 Methods of analysis of azithromycin and related substances

Various methods have been employed for the analysis of azithromycin. These methods exploit the physicochemical properties of the compound. Some of the methods that have been described in literature for the analysis of AZT are microbiological, electroanalytical, spectrophotometric, spectrofluorometric and chromatographic.

1.8.1 Microbiological methods

Microbiological assays are employed for potency estimation of antibiotics in biological fluids and quantitation of the *in vitro* antibacterial activity of these agents in pharmaceutical dosage forms. Microbiological estimation of potency is performed by comparing a dose of an antibiotic that is known to inhibit the growth of an appropriate selection of microorganisms with a dose of a standard sample of known potency that is able to produce the same degree of inhibition.

Lingerfelt *et al.* [53] determined the activity of AZT using zone of inhibition to determine the activity of azithromycin with *Staphylococcus aureus* as the reference microorganism. Turcinov *et al.* also carried out a microbiological diffusion assay using an eight by eight (8×8) Latin Square design to determine the potency of azithromycin with different microorganisms and conditions, so as to standardize the method and to select a suitable test organism for the analysis of azithromycin in formulations and biological fluids [54]. The study recommended *Sarcina lutea, Bacillus pumilus* and *Escherichia coli* as the test organisms for the analysis of

azithromycin, since they responded with satisfactory precision and accuracy for such analyses [54]. The USP [55] details an official method for the estimation of erythromycin potency using the test organism *Sarcina lutea* for potency determination by diffusion method on 8×8 Latin Square and Petri dishes while the BP [56] utilizes *Bacillus pumilus.* Turcinov *et al.* [54] compared the test microorganisms recommended by the pharmacopoeias for estimation of erythromycin potency and found out that the test microorganisms were equally sensitive. *Micrococcus luteus* has been used as the test microorganism in quantitation of azithromycin in pharmaceutical dosage forms [57]. Turcinov *et al.* [54] and Breier *et al.* [57] demonstrated that a microbiological assay using an 8×8 Latin Square design or a microbiological cylinder-plate assay can be used as an alternative method for routine quality control of azithromycin in pharmaceutical formulations.

The microbiological assays discussed though relatively inexpensive, do not distinguish between the activity of the active pharmaceutical ingredient and related substances. In addition, microbiological assays have a long turn-around time and are tedious. Most drawbacks in microbiological testing arise from the lack of accuracy and precision, since the bioassay is largely dependent on culture techniques, in addition to the physiological state of the indicator microorganism.

1.8.2 Electroanalytical methods

Electroanalytical techniques may be used for estimating the potency of pharmaceutical compounds that have electroactive functionality in their structure. The two tertiary amino groups in azithromycin are easily oxidized and this oxidation of amines forms the basis for the voltametric determination of azithromycin.

Dialkylamines are oxidized forming a radical cation by loss of one electron. This anodic oxidation occurs at a glassy electrode in a buffer system. The voltage associated with the oxidation can be measured as a function of concentration of analyte. Mandić *et al.* [27] investigated the behavior of electrochemical oxidation of AZT and its derivatives and found that AZT is electroactive only in its non-protonated form and that the process of oxidation is pH dependent and irreversible [58, 59]. Nigović *et al.* [58] compared an electro-analytical assay for the determination of AZT in pharmaceutical formulations with the HPLC method described in the USP [55] and found out that there was no significant difference between the performance of the two methods in terms of selectivity and accuracy.

A more sensitive validated method for the determination of azithromycin in dosage forms such as powders for oral suspension, capsules and biological samples, using square-wave anodic adsorptive stripping voltammetry has been reported [59]. Adsorptive stripping involves the electrochemical pretreatment of a carbon electrode before differential pulse voltammetry measurements are made. Azithromycin can be adsorbed and accumulated on the pretreated carbon electrodes, which accounts for the high sensitivity of this technique for the determination of azithromycin [59].

Voltametric methods are relatively simple, fast, inexpensive and sensitive especially in stability studies of azithromycin in solutions of different pH and in biological sample analysis, in which a low limit of quantitation must be achieved [58, 60]. However, the adsorption of the drug on the electrode surface is not sufficiently strong for significant adsorptive accumulation and, hence, it has not been analytically useful. Also adequate precision in the voltametric signal is difficult to

obtain unless the electrode surface is cleaned and pretreated by an activation procedure before each measurement. Additionally, voltametric methods cannot selectively determine AZT in the presence of its related substances.

1.8.3 Spectrophotometric methods

Azithromycin lacks sufficient chromophoric groups to enable the compound to be determined via direct spectrophotometry. It absorbs UV radiation at short wavelengths at which matrix interference from the solvents and air exists. Spectrophotometric determination is thus achieved by derivatization or complexation to form coloured products that can be determined spectrophotometrically. Azithromycin has been determined spectrophotometrically, at absorption maxima of 542-544 nm, in bulk powders, pharmaceutical formulations and spiked biological fluids by formation of a binary complex with eosin Y in aqeous buffered medium [61]. Azithromycin has also been analysed spectrophometrically based on the formation of an ion-pair with Mo (V)-thiocyanate followed by extraction with dichloroethane. The orange ion-association complex exhibits maximum absorbance at 469 nm. The method has been applied in assay of azithromycin formulations [62].

1.8.4 Spectrofluorimetric methods

Spectrofluorimetric methods exploit the presence of a fluorophore in the compound of interest or the analytical procedure must be designed in such a way that an oxidizing agent is used to facilitate the formation of a fluorescent product. Spectrofluorimetric analysis of azithromycin depends on the oxidation of the macrolide antibiotic with ceric ammonium sulphate in the presence of sulphuric acid and measurement of the resultant fluorescence of cerium (Ce) III [63]. Song *et al.* [64] developed a chemiluminescence method combined with a flow injection technique for the analysis of azithomycin. The primary aim of the method was to improve

sensitivity and shorten analytical run times. Luminol and hydrogen peroxide were used to generate chemiluminescence. The chemiluminescence of azithromycin is a result of the luminol-hydrogen structure. The chemiluminescence intensity was linear over the range of 0.1 pg/ml-1.0 ng/ml. This method is the most sensitive method reported thus far and was successfully applied to the analysis of AZT in pharmaceutical dosage forms and in biological matrices such as plasma and urine.

1.8.5 Thin layer chromatography

Thin layer chromatography (TLC), a planar adsorption chromatographic technique, is well suited for exploring the best combination of the mobile phase and stationary phase for a given sample matrix. It can be used as a preliminary test for the visualization of unknown compounds that might not be detected by liquid chromatographic (LC) detectors [65]. Visualization is made possible by the use of specific spray reagents that highlight the sample spots/bands separated by TLC.

Thin layer chromatography has been utilized in identification of azithromycin and control of related substances. The method was found to selectively and accurately estimate azithromycin in raw materials and drug products. The method was accurate and precise for purity testing, stability testing and the determination of content uniformity of commercially available AZT products [66].

1.8.6 Capillary electrophoresis

Porous monodisperse poly (glycidylmethacrylate-divinylbenzene) microspheres have proved to be well suited for the separation of macrolide antibiotics such as AZT by isocratic capillary

electrochromatography [67]. Despite the high efficiency and resolution of the method, it has not been considered a routine technique in pharmaceutical laboratories due to poor precision [67].

1.8.7 High Performance Liquid Chromatography

Azithromycin has a low molar absorptivity since it lacks a suitable chromophore. This makes selective and sensitive UV detection of this compound difficult to achieve in HPLC. However, analysis of AZT using low UV wavelengths of between 210 and 215 nm has been reported [68, 69]. The use of wavelengths lower than 210 nm in order to achieve higher sensitivity results in significant interference from impurities in complex matrices such as biological fluids and/or tissues. Furthermore, the excipients employed in formulating AZT dosage forms produce baseline instability which makes use of low detection wavelengths impractical in routine analysis.

Most of the HPLC methods developed for the analysis of AZT in complex matrices use fluorescence [70, 71], mass spectrometry [72-74] or electrochemical [75-77] detection systems. HPLC-MS is sensitive and capable of quantitation of impurities and degradation products of pharmaceutical compounds at very low concentrations [74], but it requires careful tuning of the instrument. HPLC coupled with either amperometric or coulometric electrochemical detectors would be the preferred method for the sensitive detection of macrolides, since most macrolide antibiotics contain oxidisable tertiary amines. However, to improve on the sensitivity of electrochemical methods of detection, optimization is necessary that includes pre-concentration of samples prior to the analysis of the accumulated analyte [60]. Reported HPLC methods with fluorescence detection [70, 71] require derivatization of analytes prior to detection.

Kamau *et al*. [68] developed a LC method for the analysis of azithromycin using Waters XTerra® RP18 column at 70 °C and acetonitrile-0.2M K_2HPO_4 pH 6.5-water (35:10:55) with UV detection at 215 nm. This method has the disadvantage of the long retention time for some related substances. Additionally, the use of high temperature compromises column stability and longevity.

A liquid chromatographic method with UV detection for determining azithromycin impurities in tablets has been reported whereby a linear gradient elution was employed. Detection was performed using UV spectroscopy at 210 nm [28]. The chromatographic column was a C18 column (Phenomenex Synergi® MAX-RP 4 εm, 250×4.60 mm) maintained at 50 °C. Six impurities were separated and identified with five out of the six impurities quantified with reasonable accuracy and precision.

Shepard *et al*. [78] used both coulometric and amperometric methods for the detection of azithromycin in biological samples. The optimized conditions were alkyl phenyl or polymer coated alumina (ɔRP-1) column with neutral or alkaline pH mobile phases. The coulometric method has since been used by a number of investigators to characterise the pharmacokinetics of azithromycin in humans [79-81]. A sensitive HPLC method using a C18 stationary phase with atmospheric pressure chemical ionisation mass spectrometry (APCI-MS) that requires a small sample volume of approximately 50 εl has been developed for analysis of AZT [72].

The USP [55] prescribes the use of a mobile phase with a pH 11 which requires the use of an alumina column which is expensive and difficult to obtain commercially. Also the USP method employs electrochemical detection which is not commonly available. This method also presents

the challenge of peak tailing for the main component and the apolar components. The high pH utilized reduces the lifespan of most silica based columns.

The BP [56] recommends a liquid chromatographic method that utilizes a mobile phase with pH 8 which requires an octadecylsilyl vinyl polymer as the stationary phase with UV detection at 210 nm. The high pH employed shortens the lifespan of the column. The recommended polymer column is expensive and not easily available.

The pharmacopoeial methods for the quantitative analysis of azithromycin require ɔ-bonded alumina columns, elevated pH and electrochemical detection. These chromatographic conditions are required for the analysis of AZT and other macrolide antibiotics to improve peak shape and resolution. These poor peak parameters are largely due to the basic amines which contribute to the poor peak shape at elevated pH and the low capacity factor at reduced pH. The poor peak shape at elevated pH is most likely due to amine interaction with residual silanols whilst the poor retention at low pH is due to ionization of the analyte whereby the ionized form is poorly retained.

1.9 Study justification

Azithromycin plays an important role in the management of respiratory tract infections, otitis media, skin and soft tissue infections, uncomplicated genital complications and non-gonoccoccal urethritis, mild to moderate typhoid due to multiple antibacterial-resistant micro-organisms, Lyme disease, prophylaxis of group A streptococcal infection and trachoma. Its use in the management of atypical infections for example atypical pneumonia is particularly important in immuno-compromised patients. This central role of the drug in infection management is evidenced by listing the drug by the World Health Organization and the MoH, Kenya, as an essential drug. In Kenya, the drug is listed as a second line antibiotic for the management of trachoma. The clinical usefulness of this drug is exemplified by the many azithromycin products available in the Kenyan market.

The existing methods for the analysis of azithromycin have various drawbacks. The microbiological assay methods suffer from poor reproducibility, low accuracy and the inability to selectively determine the active pharmaceutical ingredient, azithromycin, in the presence of related compounds. Also the current pharmacopoeial liquid chromatographic methods for the analysis of azithromycin utilize high pH and temperature limiting the use of traditional silica based columns. This calls for the use of the pH and temperature stable strategies to improve on peak symmetry, column longevity and stability. Thus there is the need for a simple, relatively cheap, isocratic and validated liquid chromatographic method for the analysis of azithromycin and its related substances, impurities and degradation products with UV detection. Such a method should be applicable for routine quality control and market surveillance of azithromycin bulk samples and its formulations.

1.10 Objectives

1.10.1 General objectives

To develop a simple, robust, isocratic and stability indicating HPLC method for the routine analysis of azithromycin in bulk samples and formulations.

1.10.2 Specific objectives

The specific objectives of the study were to:

a. Develop and optimize a liquid chromatographic (LC) method for the analysis of azithromycin and related substances.

b. Validate the developed LC method in accordance with ICH guidelines.

c. Apply the developed method in the assay of some commercial samples of azithromycin obtained from the city of Nairobi.

d. Apply the developed method in stress testing of azithromycin.

CHAPTER TWO

METHOD DEVELOPMENT AND OPTIMIZATION

2.1 Introduction

The need to develop better analytical methods for assessment of the quality of pharmaceutical formulations is common in pharmaceutical research. In most cases the objectives are to develop faster, more accurate, cost-effective, reproducible and reliable methods that utilize commonly available equipment as well as to circumvent any shortcomings noted with routine analytical methods.

Liquid chromatography coupled with different types of detection systems, is the most extensively used chromatographic method for the analysis of macrolide antibiotics and related substances [82]. Successful separations are usually achieved by partition, adsorption or ion-exchange processes depending on the type of stationary phase and polarity of the mobile phase used.

The development of an HPLC method follows a systematic approach in order to ensure that a simple, accurate and reproducible method is obtained. The process involves selection of appropriate conditions and their optimization. These conditions include type of column packing, length and diameter of column, mobile phase composition and flow rate, oven temperature, sample amount and detection method.

In the present study, HPLC with UV detection was selected as the preferred method of analysis for AZT, in raw materials and pharmaceutical dosage forms, based on the frequency of use of methods described in literature. Quantitative analysis of AZT from a variety of sample matrices has been successfully performed using reverse phase high performance liquid chromatography (RP-HPLC).

The aim of this study was, therefore, to develop and validate a simple HPLC method using commonly available materials and equipment for the determination of AZT in bulk samples and various dosage forms.

2.2 Experimental

2.2.1 Reagents, chemicals and solvents

Analytical grade monobasic potassium phosphate, dibasic potassium phosphate and orthophosphoric acid were from Loba Chemie Pvt. Ltd. (Mumbai, India) while triethylamine and triethanolamine were from BDH Laboratory Supplies (Poole, UK). Tert-butylammonium hydroxide (TBAOH) was from Sigma-Aldrich (Steinheim, Germany) while potassium bromide from Merck (Darmstadt, Germany).

Acetonitrile (Scharlau, Barcelona, Spain) was of HPLC grade. Purified water was prepared using distillation by GFL distillation machine type 2001/4 (Gessellschaft fur Labortechnik mbH, Burgedel, Germany).

2.2.2 Azithromycin and related substances

Azithromycin working standard (potency=96.3% w/w) was donated by the Drug Analysis and Research Unit (DARU) of the Department of Pharmaceutical Chemistry, University of Nairobi.

The working standards of the related substances, erythromycin A oxime, erythromycin A enol ether, N-demethylazithromycin, decladinosylazithromycin and azathromycin standards were purchased from Pfizer Inc. (Groton, Connecticut, U.S.A.).

2.2.3 Equipment

2.2.3.1 Melting point apparatus

The melting points of the related substances were determined using previously calibrated B-540 Buchi melting point apparatus (Buchi Labortechnik AG, Flawil, Switzerland).

2.2.3.2 Ultra-violet spectrophotometer

A double beam T90+ UV/VIS spectrophotometer supported by the UVWIN software version 5.2.0 (PG Instruments, Leicestershire, UK) and quartz cuvettes of path length 1 cm, was used to obtain UV spectra of the working standards.

2.2.3.3 Infra-red spectrophotometer

A Shimadzu IR Prestige 21 Fourier Transform Infra-red (FTIR) spectrophotometer (Shimadzu Corp., Kyoto, Japan) operating on IRSolution software Ver. 1.3 was used to record the infra-red spectra of azithromycin and the five related substances. Samples were compressed in potassium bromide into discs using a manually operated hydraulic pellet press (Perkin Elmer GmbH, Uberlingen, Germany).

2.2.3.4 Liquid chromatograph

A Shimadzu high performance liquid chromatographic (HPLC) system (Shimadzu Corp., Kyoto, Japan) was used during the study. The system was supported by a CBM-20A (S/N: L20234812629) Prominence communication bus module as the system controller and an LCSolutions software Ver. 1.22, SP1 and equipped with an SPD-M20A (S/N: L20154806698) Prominence UV/Visible photo array diode detector equipped with a deuterium lamp for ultraviolet and a tungsten lamp for the visible region. A LC-20AD (S/N: L20264807800) Prominence solvent delivery system with a dual-plunger tandem-flow solvent delivery system and a SIL-20AC (S/N: L201748) Prominence autosampler were a part of the HPLC system. The temperature was controlled using a CTO-M20AC (S/N: L202148) column oven with a block heating thermostatic chamber and a preheater system. All mobile phase preparations were degassed using a Power sonic 410 bench-top ultrasonic bath (Daihan Labtech Ltd., Kyonggi-Do, Korea).

2.3 Characterization of working standards

2.3.1 Melting points of the working standards

The melting point of each of the working standards (Table 2.1) was determined for preliminary identification whereby the experimental values were compared against those reported in literature. The melting point values obtained were within the range reported in literature. This provided preliminary evidence for the identity of the compounds.

Table 2.1: Melting points of working standards

Compound	Experimental m.p. value (°C)	Literature m.p. value (°C)
Azithromycin dihydrate	134-137	134-141
Erythromycin A oxime	156	156
Erythromycin A imino ether	138	135-150
Azathromycin	129-131	128-131
N-demethylazithromycin	144	142-144
Decladinosylazithromycin	119	210

m.p. = Melting point

2.3.2 Infra-red absorption spectra

The BP and USP describe three main methods for the preparation of solids for IR scanning. These methods include the use of Nujol mull, potassium bromide (KBr) press disks or glassy film deposits. The KBr method was used to obtain the IR spectrum of azithromycin and five of its related substances in the range of 4000-400 cm^{-1} using a Shimadzu IR Prestige 21 Fourier Transform Infra-red (FTIR) spectrophotometer (Shimadzu Corp., Kyoto, Japan). For this purpose, 1 mm thick potassium bromide pellet disks containing 1% w/w of the analyte compounds were made by triturating the analyte compound with 150 mg of KBr.

The spectra obtained were matched peak for peak with those published in literature and showed that they were concordant. This provided further evidence on the identity of the compounds. The IR spectra of azithromycin dihydrate and its related substances is shown in Appendices 1-6 and the relevant band assignments for AZT are shown in Table 2.2.

Table 2.2: Characteristic wave number assignment for azithromycin

Wave number (cm-1)	Assignment
3495	OH stretching (broad-intermolecular hydrogen bonding)
2941	C-H (aliphatic) stretching vibration
1710	C=O Carbonyl ester stretch
1450	CH_3-O (alkyl ether)
1375	CH_2-O (alkyl ether)
1165	C-O-C asymmetrical stretching (aliphatic ethers)
1050	C-O-C symmetrical stretching (aliphatic ethers)

2.3.3 Ultra-violet absorption spectra

Ultra-violet spectra of azithromycin and five of its related compounds separately dissolved in methanol to yield concentrations of 0.01 mg/ml were scanned over the wavelength range 200-400 nm using 1 cm quartz cuvettes. The scans obtained (Appendix 7-12) showed that neither azithromycin nor its related substances showed significant absorption in the UV range.

2.3.4 Purity of azithromycin and related substances

Azithromycin and the five related substances were used on ‘as it is basis’. The % purity as per label claim of each compound is as tabulated in Table 2.3. The purity of the related substances was not ascertained as it was not possible to obtain the primary chemical reference standards for the related substances.

Table 2.3: Purity of the working standards

Name of compound	Percent purity
Azithromycin	96.3
Azathromycin	89.2
N-demethylazithromycin	90.0
Erythromycin A oxime	85.0
Erythromycin A imino ether	80.0
Decladinosylazithromycin	95.0

2.4 Method development and optimization

2.4.1 Column selection

The selection of a high-performance column is essential for the development of a rugged and reproducible HPLC method for drug analysis. The choice of an analytical column depends on the physicochemical properties of the analyte such as solubility, molecular weight and/or its ionic state [83]. The retention of a drug on an HPLC column is also a function of the column packing material and column dimensions [75].

The efficiency of a packed column increases as the particle size of the stationary phase decreases [84]. Typical particle size diameter ranges for HPLC columns are between 3 ε m-10 ε m. Smaller particles result in a higher efficiency and better sensitivity. However, these small particles generate higher column backpressures compared to columns packed with larger particle sizes [84]. The sample capacity of a column increases with volume of the stationary phase, length and internal diameter of the column.

The retention times and separation efficiency of silica and alumina adsorbent phases are generally similar with polar drugs being preferentially retained [65]. Retention of a compound on reverse phase silica-based columns is primarily due to hydrophobic and silanol interactions [63]. At low mobile phase pH, basic compounds are protonated and therefore they are poorly retained and their peaks unresolved [83]. To achieve separation of basic compounds, it is necessary to raise the pH of the mobile phase. At high mobile phase pH, bases are unprotonated and their separation is mainly due to the hydrophobic interaction with the reverse phase silica. The residual silanols account for the tailing observed at high mobile phase pH.

Azithromycin is a weakly basic drug that is unstable at low pH and thus the selection of silica-based columns may be inappropriate for analysis. Retention of AZT on silica columns occurs at high pH. A useful alternative is a column packed with alumina adsorbents. Alumina columns allow for the use of high pH without compromising the stability of AZT. The USP [55] has described a separation using a ɔ-alumina column with a mobile phase of pH 11.0. However, silica columns have many advantages over ɔ-alumina columns. The main advantages include a higher sample loading capacity, a low potential for unwanted reactions during separation and availability in a wide range of chromatographically useful forms [65, 85]. Polymer encapsulation, embedded phases and base-deactivation are alternative stationary phases [86]. Base deactivated columns are packed with stationary phases that are fully hydroxylated and are of high purity and thus have reduced silica acidity. Base-deactivated columns are claimed to be superior to standard silica based stationary phases as a result of the potential reduced silanol-solute interactions [85].

Manipulation of mobile phase composition and the use of intermediate pH [65, 85] is a strategy employed when using silica-based columns. This provides better separation on silica-based columns compared to alumina-based columns with mobile phases of extreme pH. The main drawback in the analysis of AZT is the interaction that occurs between the protonated amine functional group and the free silanol functional groups which results in tailing of the peaks. The protection of the silanol groups with other organic functional groups, such as an alkyl group, has shown improved peak symmetry [83, 84]. The peak symmetry of AZT was markedly improved when the silanol groups were replaced with either amino or methyl functional groups [65, 86]. Examples of such columns that are commercially available include Waters XTerra® and Phenomenex Gemini®.

Waters XTerra® is a hybrid, reverse-phase C18 column of dimensions 25 cm length and 4.6 mm internal diameter with a particle size of 5 εm and pore size of 110 Å. It was selected for method development based on the wide pH stability range claimed by the manufacturer (1-12). The pH stability is as a result of the modification of surface silanol groups by insertion of methyl bridges between neighbouring silanol groups. The wide pH range enables analysis of acidic and basic compounds unlike conventional C18 columns that are stable over the pH range of 2-8. The column is claimed to be chemically and thermally stable. Hybrid columns such as Waters XTerra® compare favourably with the polymer columns that are recommended by compendia for the analysis of AZT owing to their superior peak symmetry.

2.4.2 Selection of detection wavelength

The UV spectra of the working standards revealed that AZT and its related substances exhibit poor absorption in the wavelength range 200-400 nm. The absence of a specific chromophore in

AZT and its related substances necessitates that detection can only be achieved at low wavelengths. Many matrix components and solvents absorb UV irradiation at low wavelengths and they may interfere with the analysis of the target compound. Consequently, the detection wavelength was fixed at 215 nm. The detection of azithromycin at this wavelength has been accomplished previously [68, 69].

2.4.3 Fixed chromatographic conditions

At the onset of method development process, some chromatographic variables were fixed based on preliminary observations. These parameters included mobile phase flow rate and sample injection volume. The mobile phase flow rate was fixed at 1.0 ml/min. The flow rate impacts on the column back pressures, analysis time and mobile phase consumption. High flow rates may result in excessive backpressures that damage the column. Similarly, low flow rates result in longer analysis time. Therefore a compromise of the two extremes of flow rates is necessary for practicality. The upper pump pressure was set at 250 bars while the lower limit was set at 5 bars. Acetonitrile with a low UV cut off (190 nm) was used as organic modifier in preparation of the mobile phase and other procedures since it absorbs UV radiation far below the detection wavelength selected.

2.4.4 Preparation of the working mixture

The working mixture was prepared by accurately weighing and dissolving the working standards in a mixture of acetonitrile and water (50:50) as diluent. The concentrations of the analyte compounds in the mixture were: Azithromycin 5.0 mg/ml, 1.5 mg/ml azathromycin, 0.64 mg/ml demethylazithromycin, 0.75 mg/ml decladinosylazithromycin, 0.304 mg/ml erythromycin A oxime and 0.31 mg/ml erythromycin A imino ether (EAIE). These concentrations were preferred

since they yielded comparable peak heights among the related substances which facilitated accurate derivation of chromatographic parameters. Preliminary experiments showed that EAIE had a higher response factor compared to other impurities when working at the 5% level of related substances in the working mixture therefore it was incorporated at about 2.5% level while that of DAZT and AZA were at 10% level.

Fresh working mixture solutions were prepared after every two days since AZT and its related substances undergo degradation with time.

2.4.5 Mobile phase composition

Mobile phases were prepared by mixing appropriate volumes of the stock buffer solution with water before adjusting the pH to the desired value using the equimolar solution of the parent acid or buffer salt followed by addition of a known volume of acetonitrile. The resultant mixture was degassed in an ultra-sonic water bath for 30 min before use.

Initial chromatographic analysis of the working mixture was carried out using unbuffered mobile phase containing only a mixture of acetonitrile and water (25:75% v/v). The working mixture (20εl) was injected into the LC system. Under these conditions, no separation of the analyte compounds was achieved as shown in Figure 2.1.

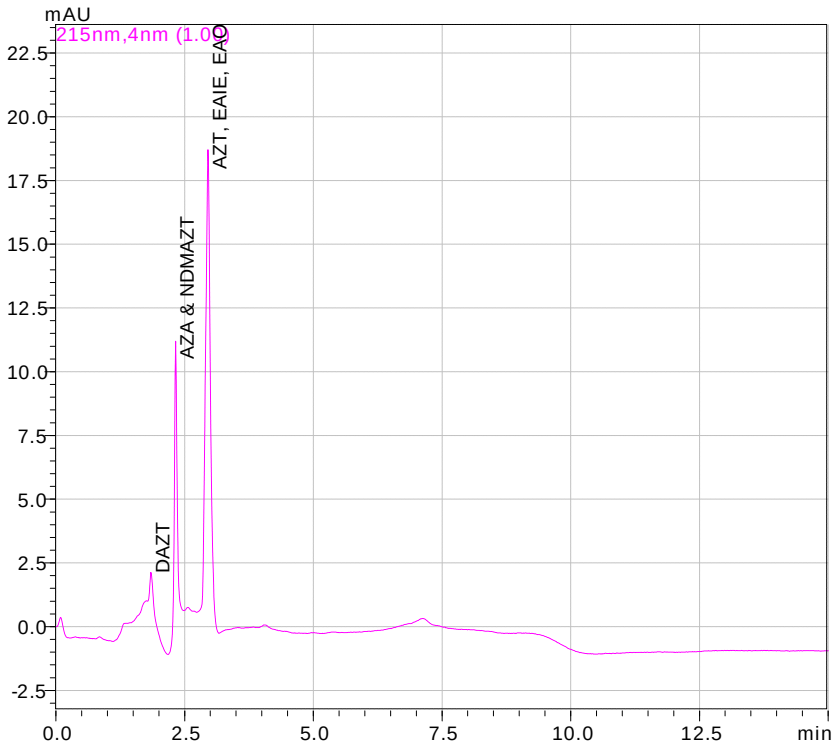


Figure 2.1: Typical chromatogram of the working mixture using unbuffered mobile phase.
Column: Waters XTerra® 5 µm C18, 250×4.6mm ID. Mobile Phase: Acetonitrile-Water (25:75, % v/v).

2.5 Influence of chromatographic factors

The incorporation of buffer and manipulation of its pH, selection of organic modifier and ion pairing agents are some of the ways of improving separation of components in HPLC. High proportions of organic modifier facilitate preferential partitioning of solutes into the mobile phase with consequent faster elution. The inclusion of a buffer and control of pH is necessary for retention of components on a column which facilitates separation of components according to their physicochemical properties.

2.5.1 Effect of inorganic buffer in the mobile phase

The influence of an inorganic buffer on the separation, tailing factor and retention time compared to that of the unbuffered mobile phase was investigated. Monobasic potassium phosphate

(KH_2PO_4) was selected since it is readily available, is commonly used in LC analyses and has a wide buffering capacity (pH 1-13). The mobile phase was prepared by mixing a stock solution of 0.1 M KH_2PO_4 with water and acetonitrile in the ratio 20:55:25 % v/v/v. The unadjusted buffer pH of the buffer solution was found to be 4.4. The chromatogram obtained showed that there was improved selectivity for EAOX and NDMAZT. Azathromycin and DAZT co-eluted with the solvent front. The introduction of buffer increased the retention of the components with the last eluting component, EAOX, having a retention time of 30 min.

The effect of adjusting pH of the phosphate buffer on the separation and symmetry of components was investigated. For this purpose mobile phases were prepared at pH 5.0, 6.0, 6.5 and 7.0. A plot of capacity factor versus pH is shown in Figure 2.2. It was also noted that an increase in the pH of the buffer improved selectivity and also caused increased retention of all the components. Improved peak shape was observed as the pH of the mobile phase was increased. At pH 7.0 there was co-elution between the critical peak pair of AZT and EAIE while at pH 6.0 there was poor resolution between AZT and NDMAZT (Figures 2.2 and 2.3). The complete loss of selectivity between AZT and EAIE at buffer pH 7, (Figure 2.3) is probably due to similarity in retention and migration behavior of the ionized forms of AZT and EAIE at neutral pH.

Therefore mobile phase consisting of 0.1M KH_2PO_4 pH 6.5 was chosen for further experiments as it exhibited good selectivity between the components especially the critical peak pair of AZT and EAIE. Figure 2.4 is a typical chromatogram obtained using buffer pH 6.5.

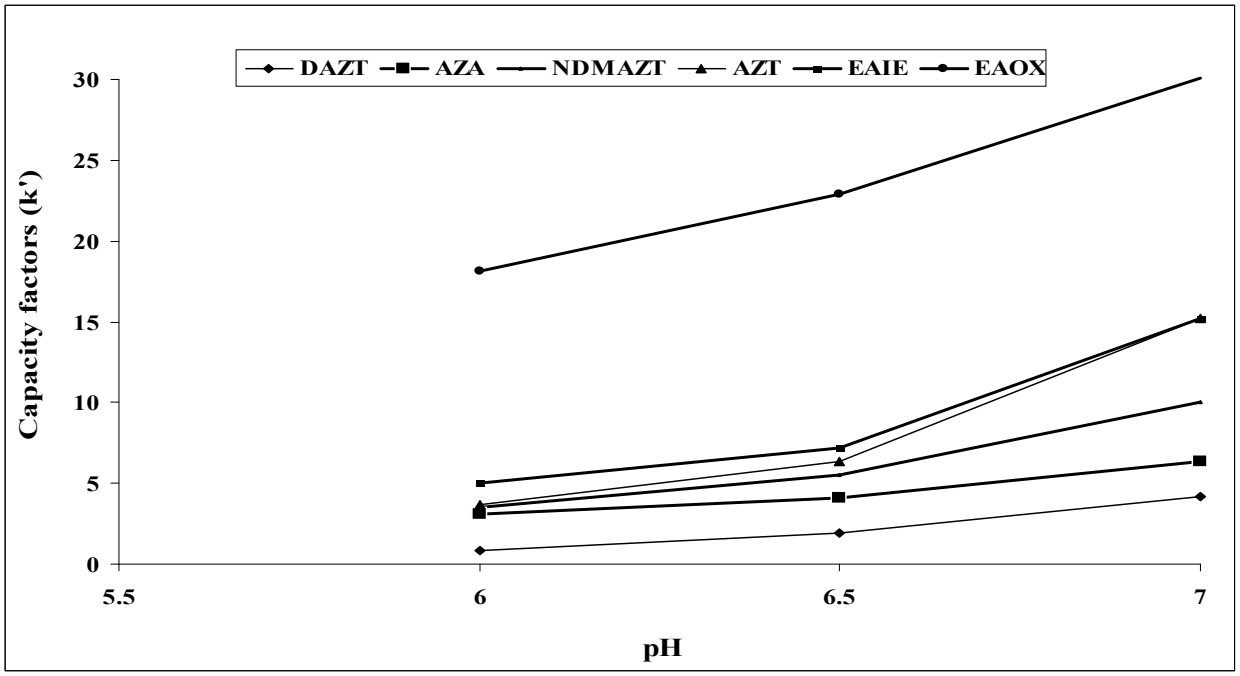


Figure 2.2: Effect of mobile phase pH on the capacity factors of components of the working mixture.

Column: Waters XTerra® 5 µm C18, 250×4.6mm ID. Mobile Phase: Acetonitrile-0.1M Potassium phosphate-Water (25:20:55, % v/v/v)

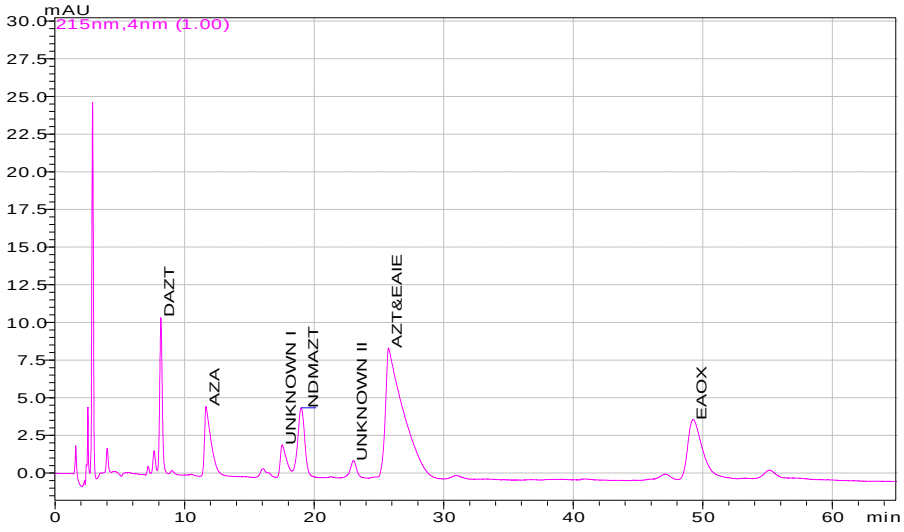


Figure 2.3: Typical chromatogram of the working mixture using potassium phosphate buffer pH 7.0.

Column: Waters XTerra® 5 µm C18, 250×4.6mm ID. Mobile Phase: Acetonitrile-0.1M Potassium phosphate-Water (25:20:55, % v/v/v).

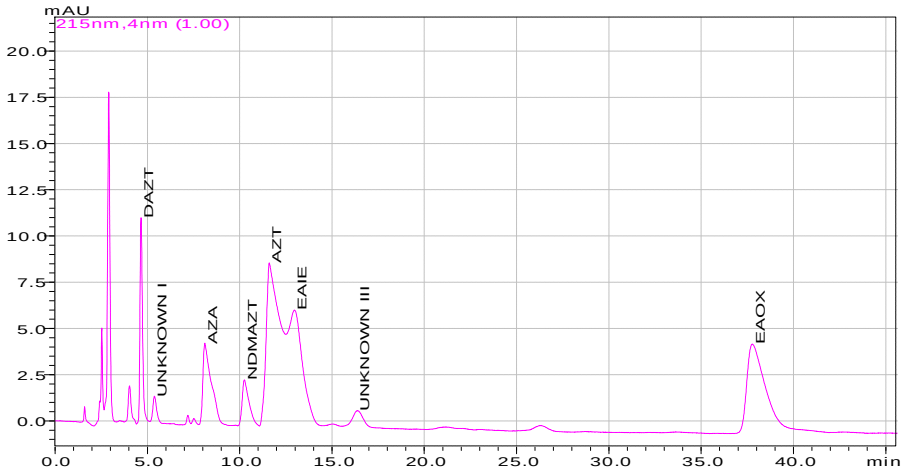


Figure 2.4: Typical chromatogram of the working mixture using potassium phosphate buffer pH 6.5.

Column: Waters XTerra® 5 µm C18, 250×4.6mm ID. Mobile Phase: Acetonitrile-0.1M Potassium phosphate-Water (25:20:55, % v/v/v).

In order to improve the separation between AZT and EAIE, the proportion of the organic modifier was reduced to 20% while maintaining pH 6.5. At these chromatographic conditions, partial resolution was achieved although the run time was increased from 60 min to 100 min as shown in Figure 2.5. Decreasing the concentration of acetonitrile, although resulting in improved separation, led to peak broadening and increased retention factors for all the components under study. However, acetonitrile concentration of 20% was adopted in the subsequent experiments.

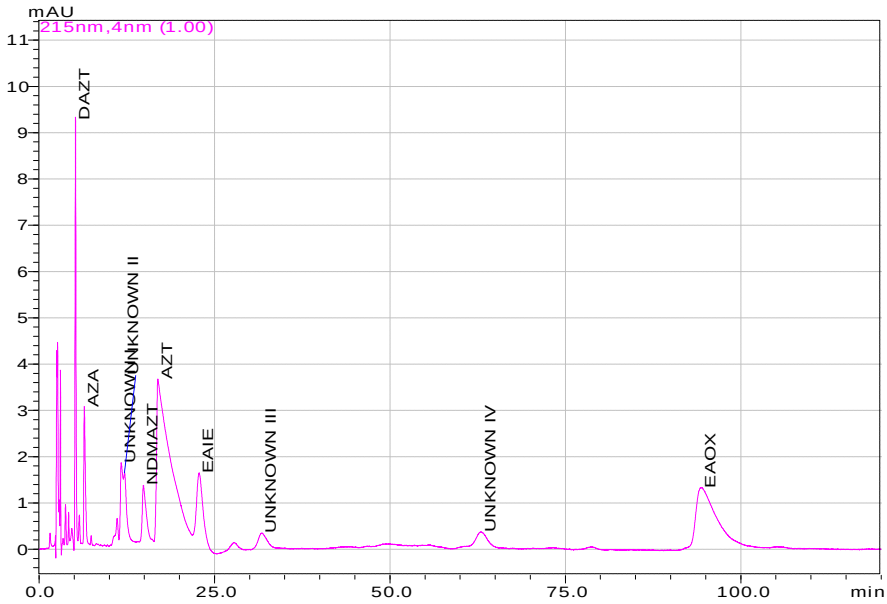


Figure 2.5: Typical chromatogram of the working mixture using potassium phosphate buffer pH 6.5 and 20% v/v acetonitrile.

Column: Waters XTerra® 5 µm C18, 250×4.6mm ID. Mobile Phase: Acetonitrile-0.1M M Potassium phosphate-Water (20:20:60, % v/v/v).

2.5.2 Effect of ion-pairing agents

Ion pairing agents are large ionic molecules that are added at low concentrations to the mobile phase to improve separation and peak symmetry. The ion-pairing agent orients itself on the stationary phase with the charged region interacting with the compounds that carry an opposite charge while the hydrophobic region interacts with the stationary phase.

Ion pairing agents are divided into anionic and cationic ion-pairing agents. The alkyl sulfonates, tertiary and quaternary ammonium salts are the commonest ion-pairing agents used in reverse-phase LC. They are incorporated into mobile phase at low concentrations Ò10 mM. These agents interact with basic and acidic analyte compounds as well as the stationary phase allowing for the separation of charged and uncharged analytes in a single run.

The incorporation of 5mM tertbutylammonium hydroxide (TBAOH) to the mobile phase resulted in baseline separation of AZT and EAIE as shown in Figure 2.6. At this point, the effect of other cationic ion pairing agents namely triethanolamine and triethylamine were investigated at an effective concentration of 5mM but none achieved baseline separation of AZT and EAIE. In addition, they had low selectivity. Therefore TBAOH was chosen for subsequent experimental work since it achieved reasonable resolution of the critical peak pair and had relatively better selectivity. It was also noted that the addition of TBAOH led to dramatic decrease in the retention time of all the components with the run time decreasing from 100 min to 45 min as shown in Figure 2.6.

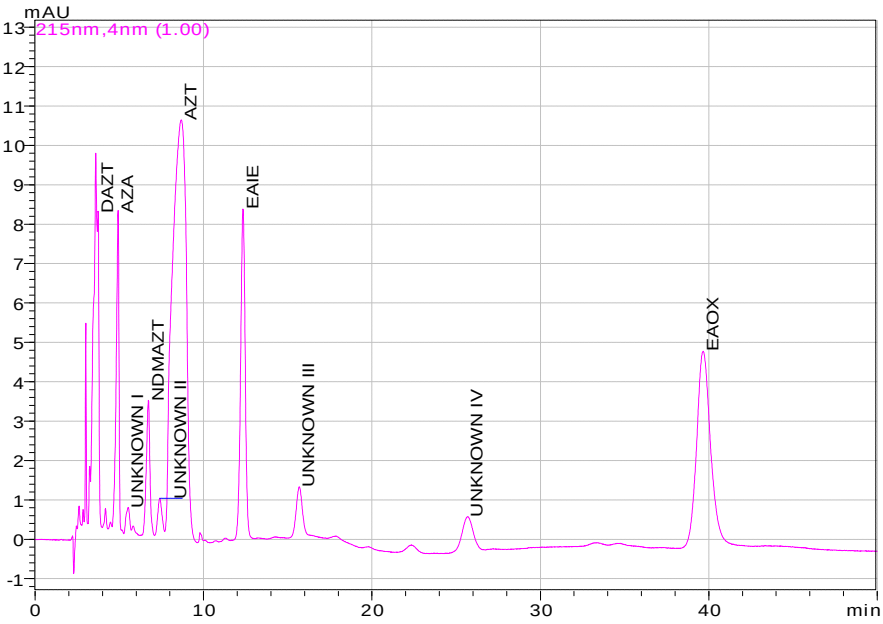


Figure 2.6: Typical chromatogram of the working mixture using TBAOH as ion pairing agent.

Column: Waters XTerra® 5 µm C18, 250×4.6mm ID. Mobile Phase: Acetonitrile-0.1M Potassium phosphate pH6.5-0.1M Tetrabutylammonium hydroxide-Water (20:20:5:55, % v/v/v/v).

2.5.3 Effect of TBAOH concentration

The effect of variation of ion pairing agent on the tailing factor of azithromycin was investigated in the range of 1-3 mM while fixing the proportions of buffer and acetonitrile at 20 % and 20 % respectively. This range was used since at higher concentrations of TBAOH, DAZT co-eluted with the solvent front. The results of the effect of varying TBAOH concentration on the peak parameters are recorded in Table 2.4.

The results obtained (Table 2.4 and Figure 2.7) justify the selection of 1mM as the optimal concentration of TBAOH since increasing the concentration of TBAOH led to fronting of AZT with reduced resolution of earlier eluting components. At concentrations above 1.5 mM TBAOH, DAZT was not resolved from the solvent front. It was observed that there was a gradual decrease in the retention time of AZT in the TBAOH concentration range of 1-2.5 mM above which it increases.

Table 2.4: Effect of ion-pairing agent on the peak parameters of AZT and EAOX

Concentration of TBAOH (mM)	Azithromycin			RT of EAOX (min)
	Retention time (min)	Asymmetry factor	Resolution of CPP	
1.0	8.346	2.159	2.540	40.327
1.5	7.754	2.501	0.859	38.043
2.0	7.740	0.861	1.401	31.336
2.5	6.891	0.625	0.783	26.760
3.0	7.433	0.647	2.469	29.290

RT=Retention time, CPP=Critical peak pair of AZT and EAIE

The increase in retention time of AZT may be attributed to saturation of silanols on the column with TBAOH and that the excess cations from the ion pairing agent are attracted by the basic amine functional groups of AZT. This resulted in increased retention of AZT in the column.

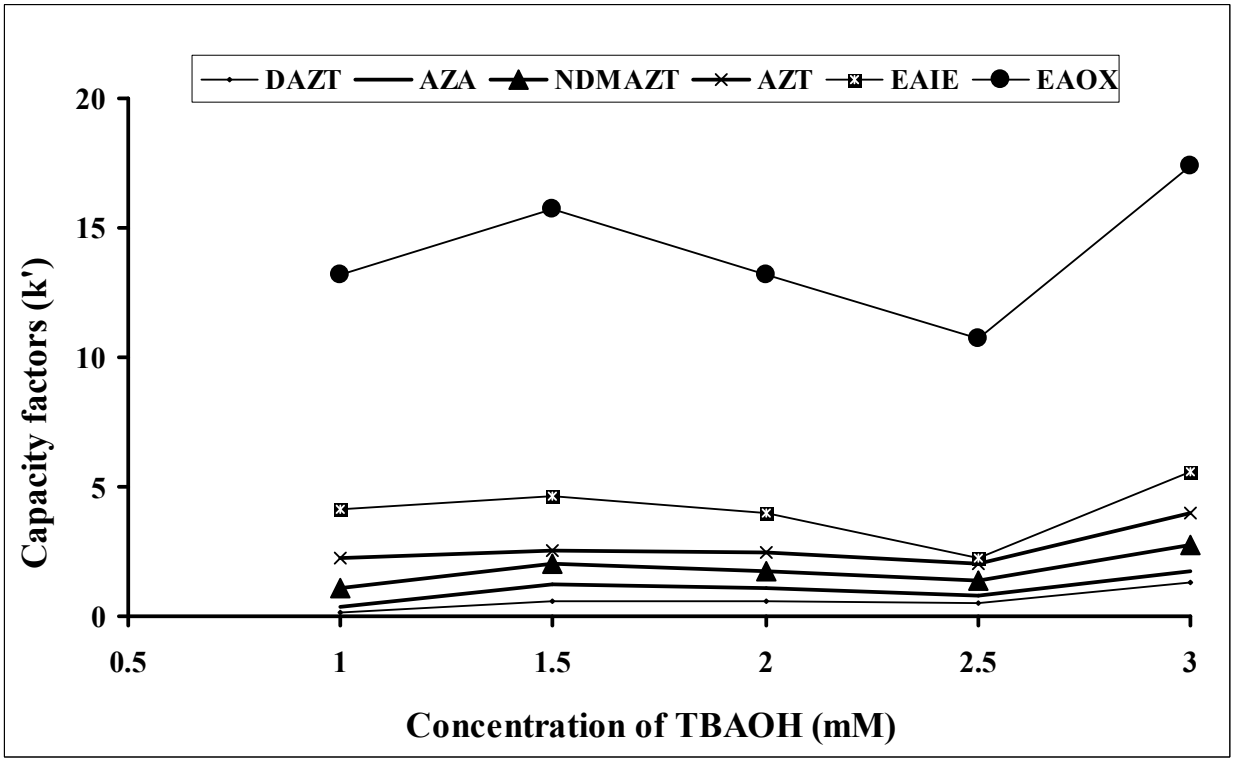


Figure 2.7: Effect of TBAOH concentration on the capacity factors of components of the working mixture.

Column: Waters XTerra® 5 µm C18, 250×4.6mm ID. Mobile Phase: Acetonitrile-0.1M Potassium phosphate pH 6.5-0.1M Tetrabutylammonium hydroxide-Water (20:20:X:55-X % v/v/v/v).

2.5.4 Effect of buffer concentration

The acetonitrile concentration and 0.1M TBAOH were maintained at 20% and 1% v/v respectively while the proportion of buffer investigated in the range of 10% to 30% at intervals of 5%. The effect of varying the proportion of the buffer is summarized in Table 2.5 and Figure 2.8. It was noted that an increase in buffer concentration led to increased retention time and peak

broadening. This observation can be attributed to the interaction of the buffer ions with the analyte resulting in increased retention factors.

As the buffer concentration was increased, the peak asymmetry factor also increased gradually (Table 2.5). At 10-15% phosphate buffer, there was fronting of AZT peak while from 20-30% there was tailing. In all ranges of the buffer concentration investigated, the peak asymmetry factor was not within limits set by the USP (2012) of 0.8-2.0. However, 15% phosphate buffer gave peak asymmetry value that was closer to unity.

Table 2.5: Effect of buffer concentration on peak parameters of AZT and EAOX

Concentration of 0.1M KH_2PO_4	Azithromycin				RT of EAOX (min)
	Retention time (min)	Asymmetry factor	Capacity factor	Resolution of CPP	
10	8.256	0.603	2.730	1.546	24.110
15	8.263	0.622	6.646	4.513	31.560
20	8.346	2.159	2.660	2.540	40.327
25	8.388	2.866	2.014	1.332	51.993
30	10.387	5.729	2.975	0.811	55.355

Column: Waters XTerra® 5 µm C18, 250×4.6mm ID. Mobile Phase: Acetonitrile-0.1M Potassium phosphate pH 6.5-0.1M Tetrabutylammonium hydroxide-Water (20:X:1:55-X, % v/v/v/v).

A buffer concentration of 15% was chosen for further experimental work since it enabled resolution of all peaks within a reasonable run time (Figure 2.8).

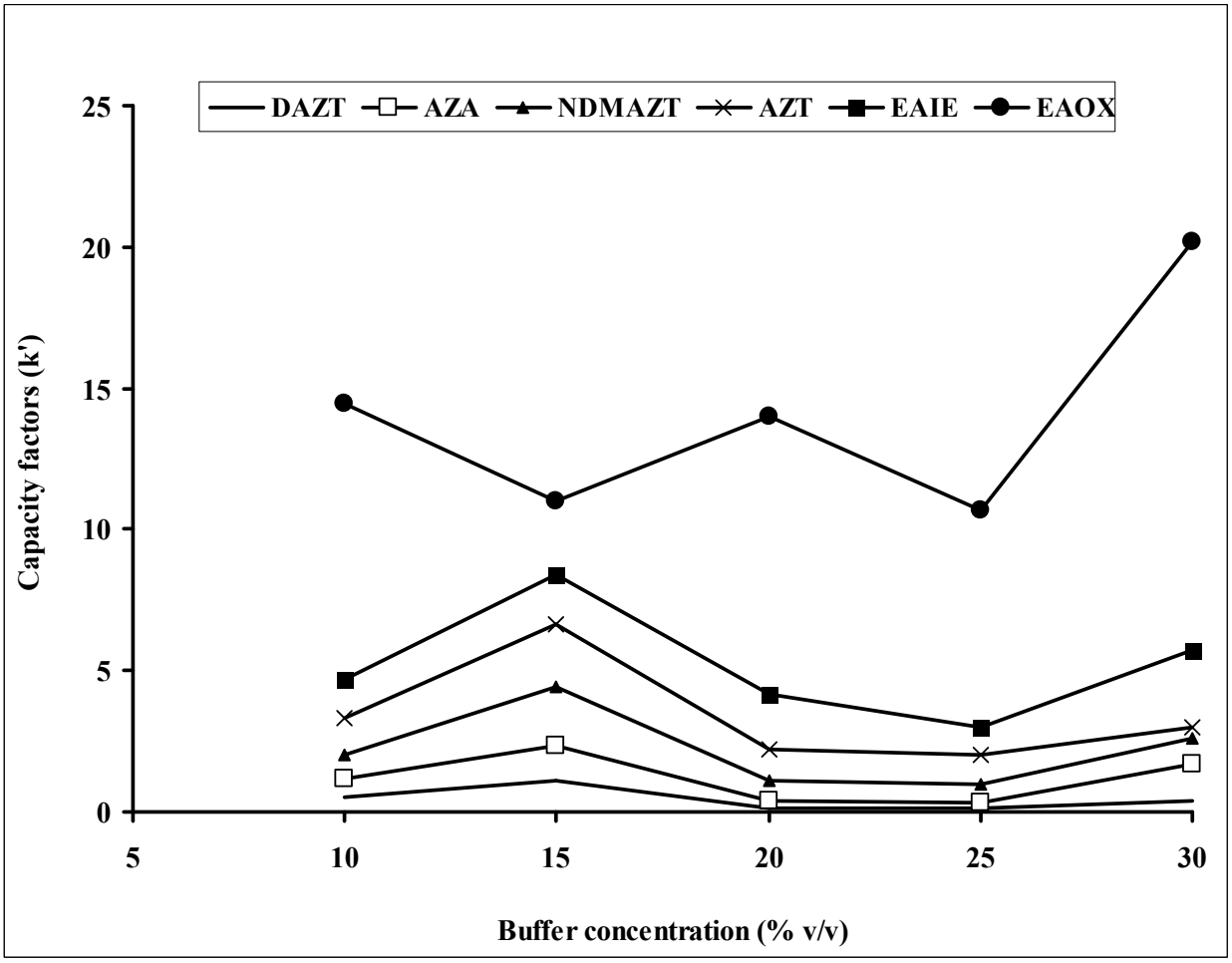


Figure 2.8: Effect of buffer concentration on the capacity factors of components of the working mixture.

Column: Waters XTerra® 5 µm C18, 250×4.6mm ID. Mobile Phase: Acetonitrile-0.1M Potassium phosphate pH 6.5-0.1M Tetrabutylammonium hydroxide-Water (20:X:1:55-X, % v/v/v/v).

2.5.5 Effect of acetonitrile concentration

The effect of the concentration of acetonitrile in the mobile phase on the retention time, capacity factors and retention factors of azithromycin was investigated. This was achieved by fixing the concentration of 0.1M KH_2PO_4 buffer at 15% and that of the ion-pairing agent at 1 mM. The effect of acetonitrile was investigated in the range of 15-25% at intervals of 5% and the results

are as shown in Table 2.6 and Figure 2.9. At 15% ACN, there was inversion of elution order of the CPP with azithromycin eluting after EAIE.

Table 2.6: Effect of acetonitrile concentration on peak parameters of AZT and EAOX

Concentration of ACN (%v/v)	AZT				RT of EAOX (min)
	Retention time (min)	Asymmetry factor	Capacity factor	Resolution of CPP	
15	35.814	1.171	37.553	14.434	90.012
20	8.346	2.159	2.660	2.540	40.327
25	6.453	0.643	1.070	2.704	19.547

Column: Waters XTerra® 5 µm C18, 250×4.6mm ID. Mobile Phase: Acetonitrile-0.1M Potassium phosphate pH6.5-0.1M Tetrabutylammonium hydroxide-Water (X:15:1:55-X, % v/v/v/v).

It was noted that increasing the proportion of acetonitrile in the mobile phase reduced the retention times and peak tailing of all the components. There was co-elution between the CPP of AZT and EAIE within the ACN concentration range of 15-20% v/v as shown in Figure 2.9. Therefore acetonitrile concentration of 25% was selected at it gave reasonable run time and peak parameters as shown in Figure 2.10.

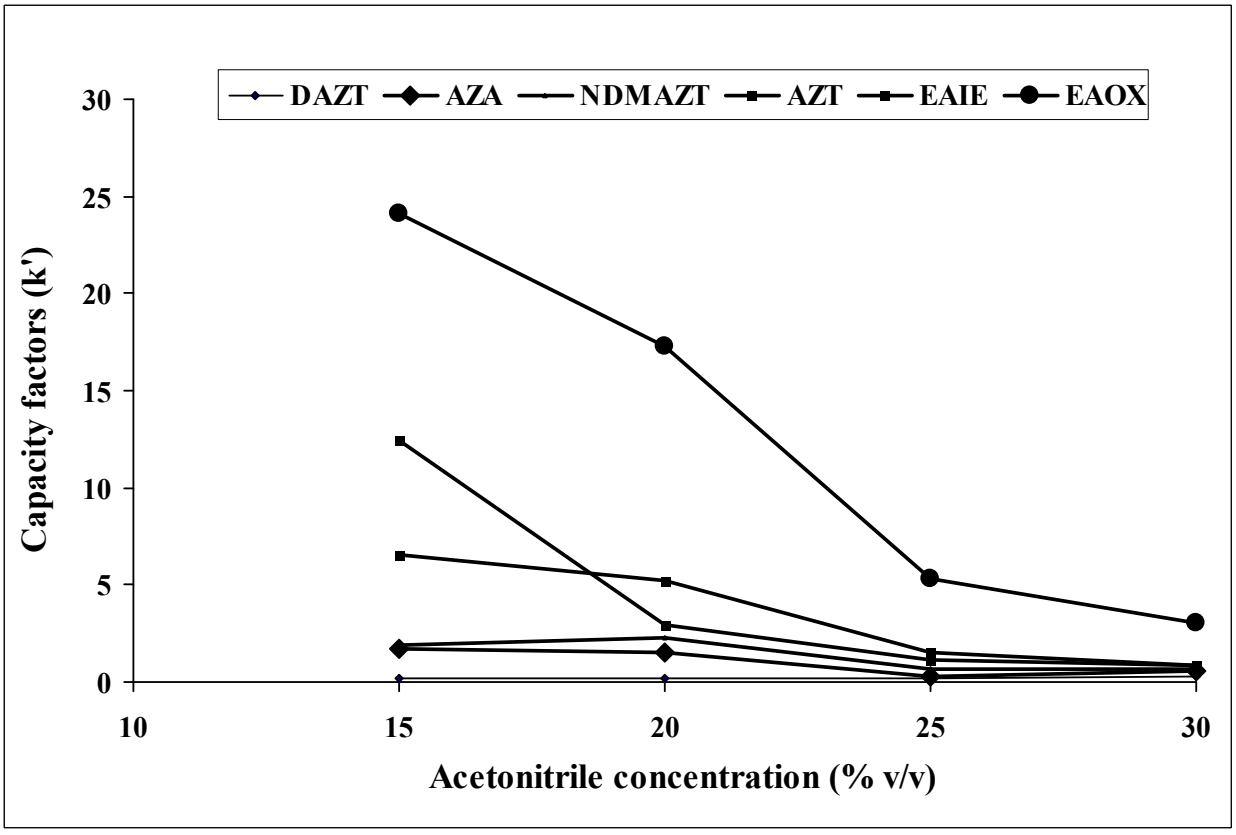


Figure 2.9: Effect of acetonitrile concentration on the capacity factors of components of the working mixture.

Column: Waters XTerra® 5 µm C18, 250×4.6mm ID. Mobile Phase: Acetonitrile-0.1M Potassium phosphate pH 6.5-0.1M Tetrabutylammonium hydroxide-Water (X:15:1:55-X, % v/v/v/v).

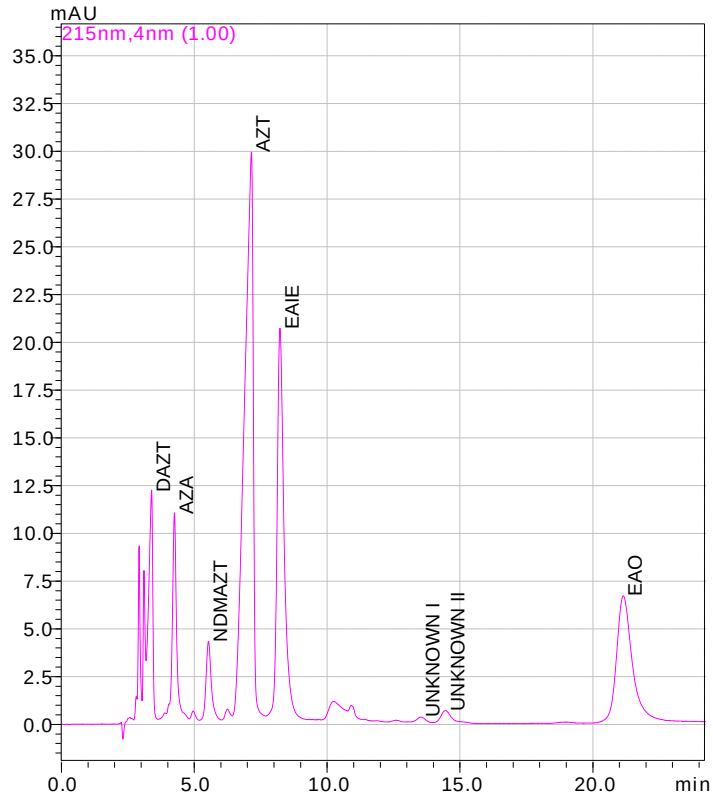


Figure 2.10: Typical chromatogram of the working mixture using potassium phosphate buffer pH 6.5 and 25% v/v acetonitrile.

Column: Waters XTerra® 5 µm C18, 250×4.6mm ID. Mobile Phase: Acetonitrile-0.1M Potassium phosphate pH 6.5-0.1M Tetrabutylammonium hydroxide-Water (25:15:1:59, % v/v/v/v).

2.5.6 Effect of column temperature

Temperature affects the separation of components in a mixture in LC. Temperature control is necessary in HPLC for reproducibility of retention times and ruggedness. An increase in temperature lowers the viscosity and density of mobile phase liquids and therefore reduces the column back pressures. Elevated temperatures lead to faster elution of components as a result of temperature-induced increase in mass transfer of analytes in the column and this infuences productivity. However, extremely high temperatures above 60 °C damage columns especially if the pH is above 7.0 for silica based columns. Furthermore, some compounds are thermally

unstable at elevated temperatures. Initially the temperature was fixed at 40 °C while investigating the effects of organic modifier, buffer and ion-pairing agent on the separation and symmetry of azithromycin and its related substances. The temperature range investigated was 41-45°C.

An increase in oven temperature led to a slight increase in retention of azithromycin and its related compounds (Table 2.7). This is a property observed with all macrolides which may be due to reduced solvation of the compounds at elevated temperature [87].

Table 2.7: Effect of temperature on peak parameters of azithromycin

Temperature (°C)	AZT			
	Retention time (min)	Asymmetry factor	Capacity factor	Resolution of CPP
41	7.834	0.685	1.169	3.831
43	8.075	1.123	1.210	3.576
45	8.078	1.343	1.193	3.368

Column: Waters XTerra® 5 µm C18, 250×4.6mm ID. Mobile Phase: Acetonitrile-0.1M Potassium phosphate-0.1M Tetrabutylammonium hydroxide-Water (25:15:1:59, % v/v/v/v).

At 40 °C, the retention time of AZT was 6.45 min while the peak asymmetry and capacity factors were 0.64 and 1.07 respectively. Increasing the temperature from 40 °C to 45 °C led to an increase in the selectivity between DAZT and solvent front since organic impurities present did not respond to temperature changes. This aided in resolution of the solvent peak from DAZT. At 46 °C, there was co-elution of AZA with DAZT and also AZT with EAIE. A column temperature of 43 °C was selected as the optimum since it gave the highest resolution between the critical peak pair of AZT and EAIE while maintaining the selectivity of DAZT. Also 43 °C gave a peak asymmetry factor for AZT which was within the ICH recommended limits [88]. The effect of temperature on the capacity factors of components is shown in Figure 2.11.

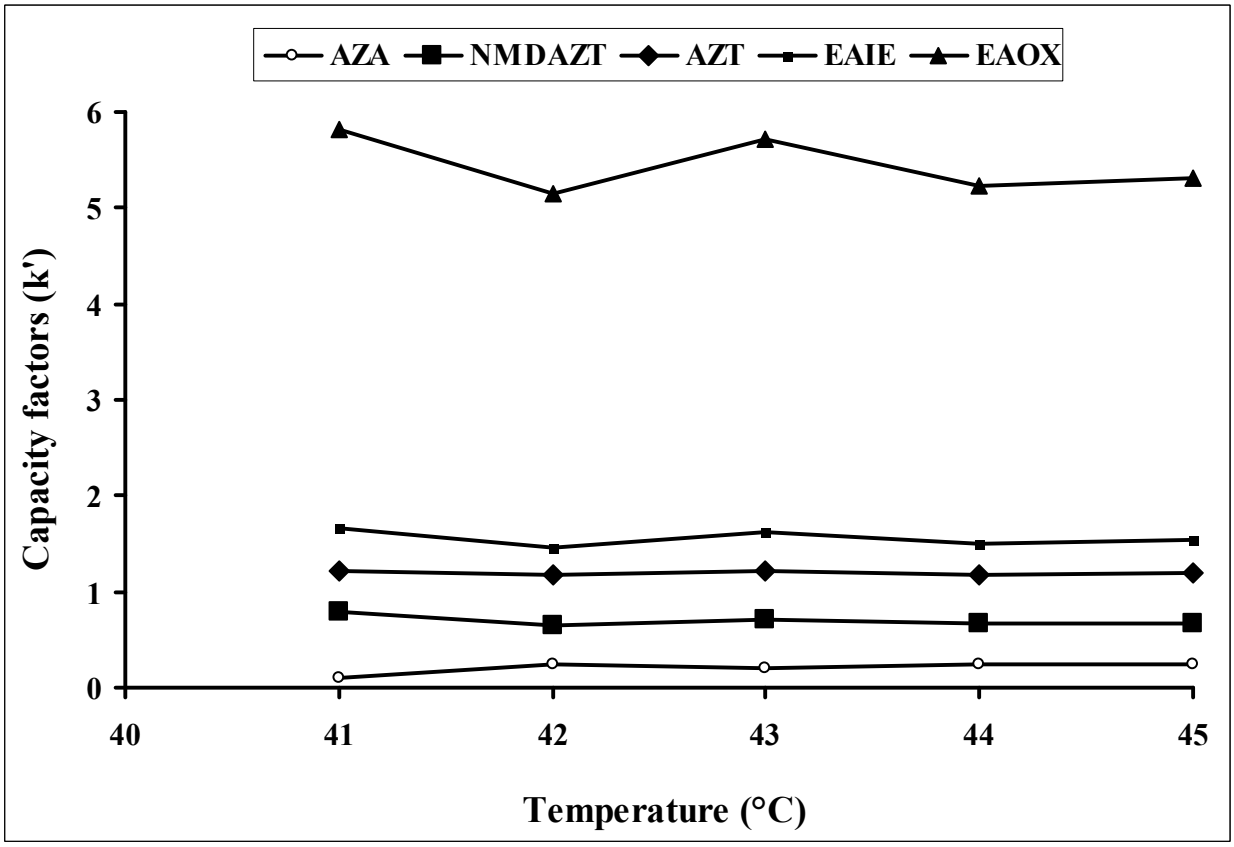


Figure 2.11: Effect of column temperature on the capacity factors of components of the working mixture.

Column: Waters XTerra® 5 µm C18, 250×4.6mm ID. Mobile Phase: Acetonitrile-0.1M Potassium phosphate pH 6.5-0.1M Tertbutylammonium hydroxide-Water (25:15:1:59, % v/v/v/v).

2.5.7 Optimized chromatographic conditions

From the data collected, optimum chromatographic parameters for separation of components were established as mobile phase comprising of acetonitrile-0.1M KH_2PO_4 pH 6.5-0.1M tetrabutyl ammonium hydroxide pH 6.5-water (25:15:1:59 % v/v/v/v) delivered at a flow rate of 1.0 ml/min. The stationary phase consisted of reverse-phase Waters XTerra® C18 column of dimensions 25 cm length and 4.6 mm internal diameter with particle size 5 µm maintained at a temperature of 43 °C with a detection wavelength of 215 nm. A typical chromatogram obtained at the optimized conditions is as shown in Figure 2.12.

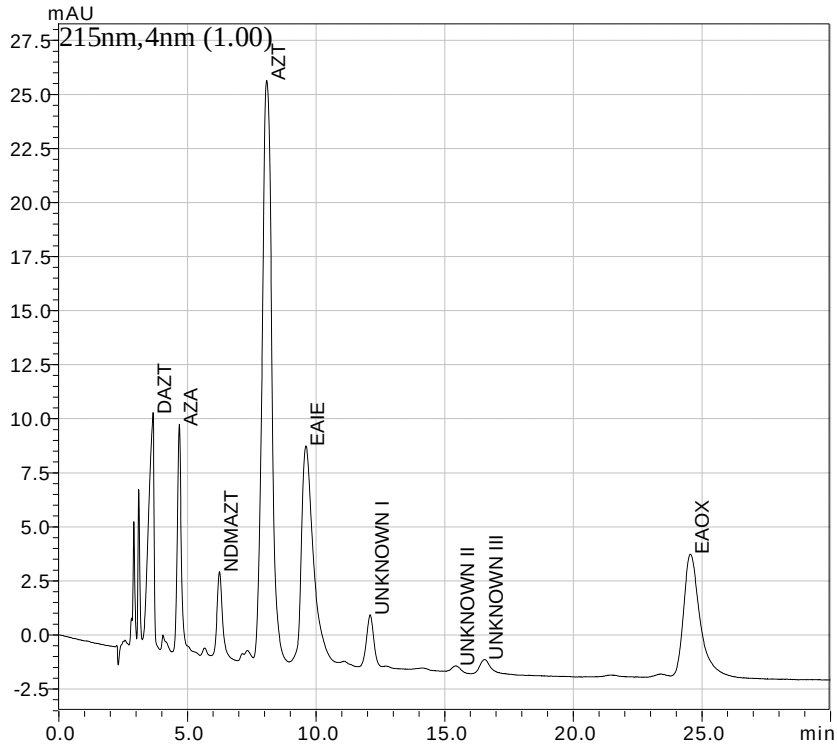


Figure 2.12: Typical chromatogram of the working mixture obtained under optimized chromatographic conditions.

Column: Waters XTerra® 5 µm C18, 250×4.6mm ID. Mobile Phase: Acetonitrile-0.1M Potassium phosphate pH 6.5-0.1M Tertbutylammonium hydroxide-Water (25:15:1:59, % v/v/v/v). Column temperature: 43 °C. Flow rate: 1.0 ml/min. Detection: 215 nm. Injection volume: 20 µl.

CHAPTER THREE

METHOD VALIDATION

3.1 Introduction

Validation is the process that establishes by laboratory studies that the performance characteristics of the method meet the requirements for the intended analytical applications. The validation of the developed method was performed in accordance with ICH guidelines [88]. The validation parameters include accuracy, precision, (repeatability, intermediate precision), specificity, detection limit, quantitation limit, linearity and range. Limit of detection and limit of quantitation were used as measures of sensitivity.

3.2 Linearity and range

Linearity is the ability of a method to elicit test results that are directly proportional to analyte concentration within a given range. Linearity is generally reported as the coefficient of determination (r^2) of the slope of the regression curve. Range is the interval between the upper and lower levels of analyte that have been demonstrated to be determined with precision, accuracy and linearity using the method. The range is normally expressed in the same units as the test results obtained by the method. A minimum of five concentration levels, along with certain minimum specified ranges are required. For assay, the minimum specified range is from 80-120% of the target concentration [88]. Acceptance criteria for linearity is that the coefficient of determination (r^2) is >0.99 for the least squares method of analysis of the regression curve.

Additionally, the coefficient of variation (CV) will not be greater than 5.0% at all concentrations of the standard solution [88].

Linearity was determined on the solutions of the azithromycin working standard within the 50-150% range. A 5.0 mg/ml solution was taken as 100%. Solutions were made by dissolving azithromycin in diluent consisting of acetonitrile and water (50:50). Appropriate dilutions of these solutions were made to yield the concentrations listed in Table 3.1. Triplicate determinations were done for each solution and peak areas recorded. The data obtained from the linearity experiments was subjected to linear regression analysis with the concentration of injected AZT standard being plotted against the peak areas obtained and the results are summarized in Table 3.1. The developed method met the ICH guidelines for linearity since r^2 was >0.99.

Table 3.1: Linearity of detector response for azithromycin

Concentration (mg/ml)	Concentration of analyte target (%)	CV of peak area
2.50	50	0.06
3.75	75	0.12
5.00	100	0.02
6.25	125	0.11
7.50	150	0.02
Equation for regression: **y=9948x-40457**		Correlation coefficient (r^2)=**0.997**

Column: Waters XTerra® 5 µm C18, 250×4.6mm ID. Mobile Phase: Acetonitrile-0.1M Potassium phosphate pH 6.5-0.1M Tertbutylammonium hydroxide-Water (25:15:1:59, % v/v/v/v). Column temperature: 43 °C. Flow rate: 1.0 ml/min. Detection: 215 nm. Injection volume: 20 µl.

3.3 Accuracy

The accuracy of an analytical procedure measures the closeness of agreement between the value, which is accepted either as a conventional true value or an accepted reference value and value found. Accuracy is a measure of exactness of an analytical method. Accuracy is measured as the percent of analyte recovered by assay, by spiking samples in a blind study [88].

Accuracy was evaluated by analyzing synthetic mixtures spiked with known quantities of azithromycin. A minimum of nine determinations over a minimum of three concentration levels covering the specified range were injected in triplicate. The mean recovery of the assay should be within 100±2.0% at each concentration over the range of 80-120% of nominal concentration [88, 89]. The results shown in Table 3.2 indicate that the developed method is accurate since the mean recovery obtained was 100.7%.

Table 3.2: Recovery of azithromycin from spiked samples

Target concentration (%)	Azithromycin				Mean recovery (%)
	Amount added (mg)	Amount recovered (mg)	Recovery (%)	Recovery (CV)	
80	4.98	4.975	99.9	0.16	100.7
100	5.08	5.171	101.8	0.20	
120	5.13	5.152	100.4	0.50	

Column: Waters XTerra® 5 µm C18, 250×4.6mm ID. Mobile Phase: Acetonitrile-0.1M Potassium phosphate pH 6.5-0.1M Tertbutylammonium hydroxide-Water (25:15:1:59, % v/v/v/v). Column temperature: 43 °C. Flow rate: 1.0 ml/min. Detection: 215 nm. Injection volume: 20 µl.

3.4 Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions [88]. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility. The precision of an analytical procedure is expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

3.4.1 Repeatability

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision. Repeatability is determined from a minimum of nine determinations covering the specified range of the procedure (for example, three levels, three repetitions each), or from a minimum of six determinations, at 100% of the test or target concentration. The coefficient of variation (CV) for replicate injections should not be greater than 1.5% [88].

The repeatability was determined by a six times injection of azithromycin working solution (5.0 mg/ml). The CV of the peak areas was 0.97% and this was within the limit recommended by ICH [88].

3.4.2 Intermediate precision

Intermediate precision expresses within-laboratories variations such as different days, different analysts and different equipment among other variables. Intermediate precision was demonstrated by injecting five replicate solutions of azithromycin working standard on day 1, 2

and 3 and the peak areas recorded in Table 3.3. The coefficient of variation was calculated and is shown in Table 3.3. In all determinations, the CV was less than 1.5 thus the HPLC method was found to be precise.

Table 3.3: Intermediate precision of the method

Concentration of azithromycin (5 mg/ml)	Peak area		
	Day 1	Day 2	Day 3
Run 1	699180	697105	700121
Run 2	700736	697314	699118
Run 3	700114	697135	699147
Run 4	699136	698152	699160
Run 5	699098	697142	700104
Average	699653	6973670	699530
SD	739.0	445.0	532.0
% RSD (CV)	0.11	0.06	0.08

Column: Waters XTerra® 5 µm C18, 250×4.6mm ID. Mobile Phase: Acetonitrile-0.1M Potassium phosphate pH 6.5-0.1M Tertbutylammonium hydroxide-Water (25:15:1:59, % v/v/v/v). Column temperature: 43 °C. Flow rate: 1.0 ml/min. Detection: 215 nm. Injection volume: 20 µl.

3.5. Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products and excipients [88]. It is a measure of the degree of interferences from such components, ensuring that a peak response is due to a single component only. Resolution, efficiency and tailing factor are measures of specificity. Resolution between azithromycin and all the components was >1.5. The efficiency of the column was >1500 theoretical plates for azithromycin peak while the tailing factor was <2 as per the ICH guidelines [88, 89]. The peak purities were determined using the photo diode array

detector for all the separated peaks and were found to between 0.9999-1.000. This confirmed that the eluted peaks did not contain any other impurity peaks or co-eluted peaks [90].

3.6 Sensitivity

Sensitivity is the ability to detect small changes in the concentration of the analyte in the sample. There are two parameters that are used to assess the sensitivity of an analytical method. These are the limit of detection (LOD) and the limit of quantitation (LOQ). The detection limit is the lowest concentration of analyte in a sample that can be detected, but not necessarily quantified, under the stated experimental conditions while the LOQ is lowest amount of analyte in a sample that can be determined with acceptable precision and accuracy under stated conditions [88]. The detection limit was determined by serially diluting 5 mg/ml AZT solution until it produced a signal to noise ratio of 3. The solution was injected in triplicate and the signal and the noise for each injection were recorded. Each signal to noise ratio (S/N) was calculated and averaged. Calculated against a sample solution of 5.0 mg/ml, the LOD was 20 ε g at a S/N ratio of 3 while the LOQ was 78 ε g at a S/N ratio of 10. The LOD and LOQ results obtained demonstrated that the developed method was sensitive.

3.7 Robustness

The ability of an analytical method to remain unaffected by small changes in parameters is defined as its robustness [55, 88]. The robustness of the developed method was determined by making small but deliberate adjustments in the optimized chromatographic factors according to ICH guidelines [88]. For purposes of determining the experimental design and data analysis, the computer software, Statgraphics Centurion XVI (StatPoint Technologies Inc., USA) was used. The software was chosen for robustness study since it allows use of minimum number of

experiments with a predictive ability over the range of the responses. The software randomizes the order of experiments to be carried out. This software also performs data analysis by generating summary statistics, impact of the various factors on the responses as well as the response surfaces.

3.7.1 Design of experiments

The Box-Wilson Design (BWD) model was applied to determine the optimum range of the chromatographic factors within which the developed HPLC method was robust. The main focus of the study was to find the optimal range in which variations on the chromatographic factors of acetonitrile concentration, buffer pH and oven temperature do not affect the method. These three factors were the only ones investigated since they had the most impact on the selectivity of the critical peak pair.

The BWD is a statistical technique that investigates the impact of the experimental variables on the response output using the central composite design (CCD) [91]. According to the central composite design the number of experiments is given by the equation:

$$2^k+2k+1$$

where k was the number of experimental factors, 2^k was the factorial components of the experiment , 2k was the standard number of experiments while 1 is the minimum number of centre experiments.

Although the model gives 15 experiments, 17 experiments were carried out. The extra two were additional centre experiments. The chromatographic variables (k) evaluated were of acetonitrile concentration, pH and temperature. Each of the experimental factors was evaluated at three levels namely low, central and high as shown in Table 3.4.

Table 3.4: Nominal values corresponding to low, central and high levels

Factor	Low level (-1)	Central level (0)	High level (+1)
pH (A)	6.0	6.5	7.0
ACN (B)	22	25	28
Temperature (C)	41	43	45

The order of the experiments was randomized with the aid of the software as shown in Table 3.5. The interactive effects of the three variables on the selectivity of the critical peak pair of EAIE and AZT were evaluated as a measure of robustness.

Table 3.5: Randomized experiments for robustness testing

Experiment No.	pH	Acetonitrile	Temperature (°C)
1	7.0	28.0	41.0
2	6.5	25.0	41.0
3	6.0	22.0	45.0
4	6.0	28.0	41.0
5	6.0	22.0	41.0
6	6.5	25.0	43.0
7	7.0	25.0	43.0
8	6.5	28.0	43.0
9	7.0	22.0	45.0
10	6.5	25.0	43.0
11	6.5	25.0	45.0
12	6.0	25.0	43.0
13	6.5	25.0	43.0
14	6.5	22.0	43.0
15	7.0	28.0	45.0
16	6.0	28.0	45.0
17	7.0	22.0	41.0

3.7.2 Standardized Pareto charts

A Pareto chart, also called a Pareto distribution diagram, is a bar graph in which values are plotted in decreasing order of relative frequency. Pareto charts quickly point out the factors that have more impact than others. Longer bars on the chart, which represent frequency, illustrate which variables have the greatest cumulative effect on a given system. Independent variables on the charts are shown on the y-axis while the dependent variables are portrayed as the heights of bars or vice versa. Therefore, Pareto charts enabled the identification of the most significant factors.

The bars on Pareto charts are displayed in order of magnitude of the effects from the largest to the smallest. The charts include a vertical line at the critical t-value for Ŭ-value of 0.05. Effects for which bars are smaller than the t-critical value were considered insignificant. Also the second order interactive effects of same variable were considered insignificant. The estimated effects of the variables and their second order interactions on the selectivity of CPP of AZT and EAIE were presented on standardized Pareto charts (Figure 3.1 and Figure 3.2).

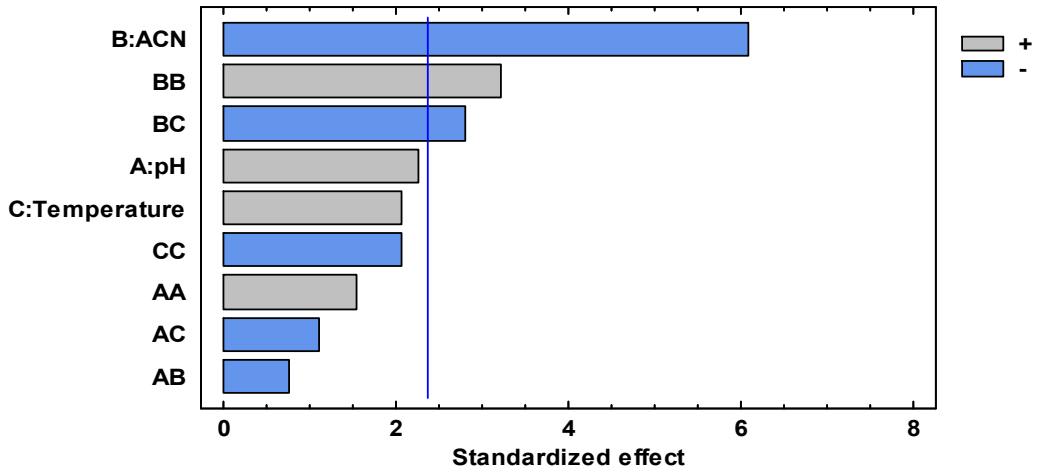


Figure 3.1: Standardized Pareto chart for EAIE

Figure 3.1 shows that acetonitrile and the interactive effects between acetonitrile concentration and temperature had the greatest impact on the capacity factor of EAIE (p=0.05) with ACN having the greatest effect followed by acetonitrile then the combination of ACN and temperature. Acetonitrile had a negative effect on the selectivity of EAIE (Figure 3.1). The interactive effect of ACN and temperature on the selectivity of EAIE is also negative while those of pH and temperature were positive. This indicates that as the acetonitrile concentration was increased, the selectivity of EAIE reduced. Another important interactive effect was that of acetonitrile and temperature. This implies that as the acetonitrile was increased and at low temperature, the selectivity of EAIE decreased.

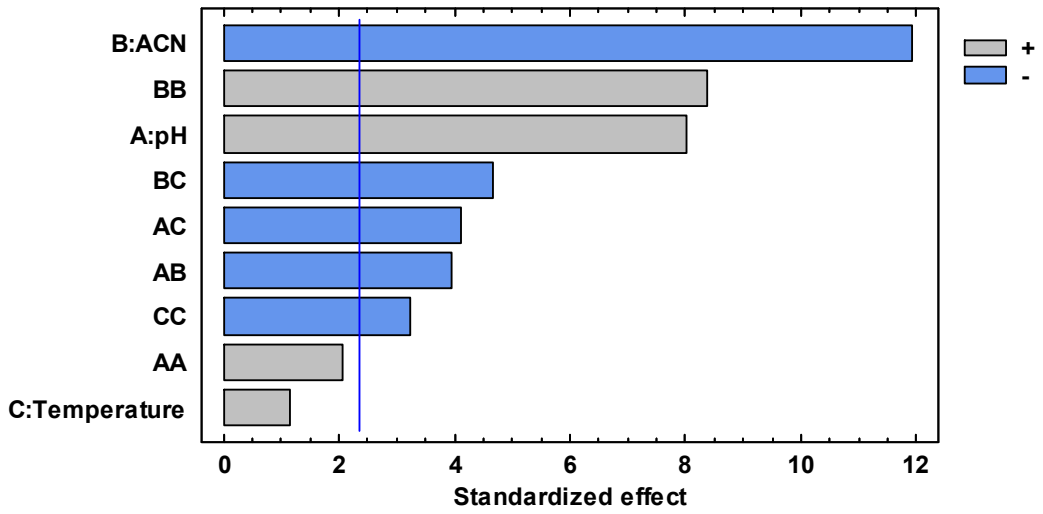


Figure 3.2: Standardized Pareto chart for AZT

Acetonitrile concentration had the biggest impact on the selectivity of AZT followed by buffer pH (p=0.05) (Figure 3.2). Acetonitrile had a negative effect on the selectivity of AZT while that of pH was positive. This implied that as the pH and acetonitrile concentration were increased they increased and reduced the selectivity of AZT respectively. Other important interactive effects were those of ACN and temperature, pH and temperature and pH and ACN. This implied that as the acetonitrile is increased and at low pH, the selectivity of AZT decreases. Figures 3.1 and 3.2 indicate that pH, acetonitrile concentration and the interactive effects between temperature and concentration of ACN were the critical factors that influence the selectivity of the critical peak pair of AZT and EAIE.

3.7.3 Response surfaces

The BWD utilizes response surfaces for the purpose of response optimization. A plot showing how the response changes with respect to changes in the factor levels is a response surface: For the purposes of plotting the response surfaces, the factors of ACN concentration, oven temperature and buffer pH were used. The capacity factors of the CPP of AZT and EAIE on the responses were measured. A plot of kNjas the function of the two most important chromatographic variables of buffer pH and acetonitrile concentration yielded response surfaces. Response surfaces were drawn for each of the components of the CPP and then overlaid onto one another to show the interactive effects (Figure 3.3).

Response surface plots that do not intersect indicate separation under the range of conditions examined while intersecting ones indicate co-elution. The corresponding response surface plots were not intersecting at any point indicating that there was no co-elution. It was noted that as the pH approached 6.0, there was poor separation between AZT and EAIE but there was an improvement as pH approached 7.0. Therefore, it is imperative to accurately measure the volume of acetonitrile and buffer pH.

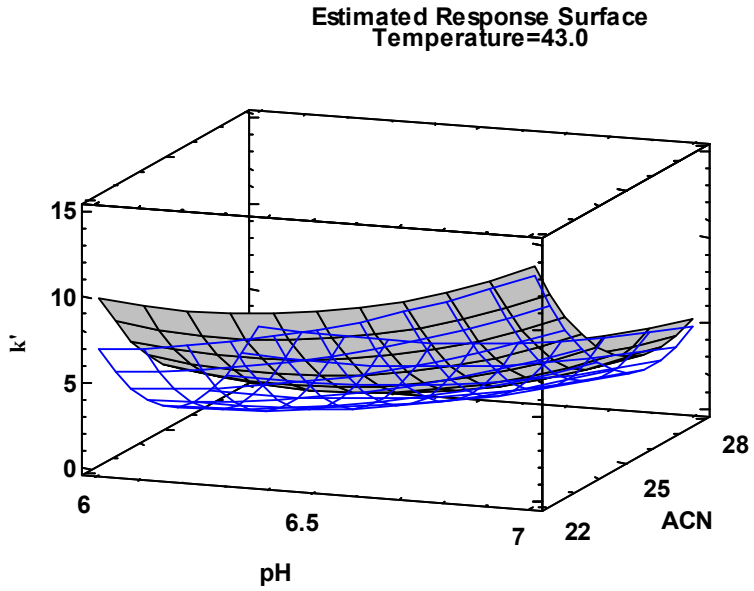


Figure 3.3: Overlaid response surfaces AZT and EAIE

3.8 Stress testing of azithromycin

The stability indicating ability of the developed method is an ICH requirement for analytical methods [88]. The developed method should demonstrate its ability to selectively quantify AZT in the presence of degradation products that may arise during drug formulation, manufacturing and/or storage. Stress testing of AZT was done by subjecting the working reference standard solution to forced degradation in acid and in hydrogen peroxide. The peak areas of AZT were monitored as a function of time and the data generated was subjected to reaction kinetic modeling using zero, first and second order integrated rate equations. The integrated rate and half life equations used are shown in Table 3.6.

Table 3.6: Integrated rate and half life equations

Order of reaction	Integrated rate equation	Half life equation
Zero	$A_t = A_o - kt$	$t_{1/2} = \frac{A_0}{2k}$
First	$\ln[A]_t = -k\,t + \ln[A]_0$	$t_{1/2} = \frac{\ln 2}{k}$
		$t_{1/2} = \frac{0{\cdot}693}{k}$
Second	$1/[A]_t = 1/[A]_0 + k\,t$	$t_{1/2} = \frac{1}{A_0 k}$

t=time, A_t=Peak area at time, t, A_0= Peak area at time, 0, k=rate constant.

3.8.1 Oxidative degradation of azithromycin

The presence of lone pairs of electrons on the amine functional group on desosamine makes AZT amenable to oxidative degradation to yield azithromycin N-oxide (Figure 3.4). This stress study was done using 0.0005% v/v H_2O_2 using the method previously described by Abuga *et al.* [92]. For this purpose, azithromycin 5 mg/ml solution was incubated in 0.0005% v/v H_2O_2 at 37 °C and the solution sampled at intervals for HPLC analysis. The peak areas of AZT were monitored for 7 h using the developed method and the areas obtained are shown in Table 3.7.

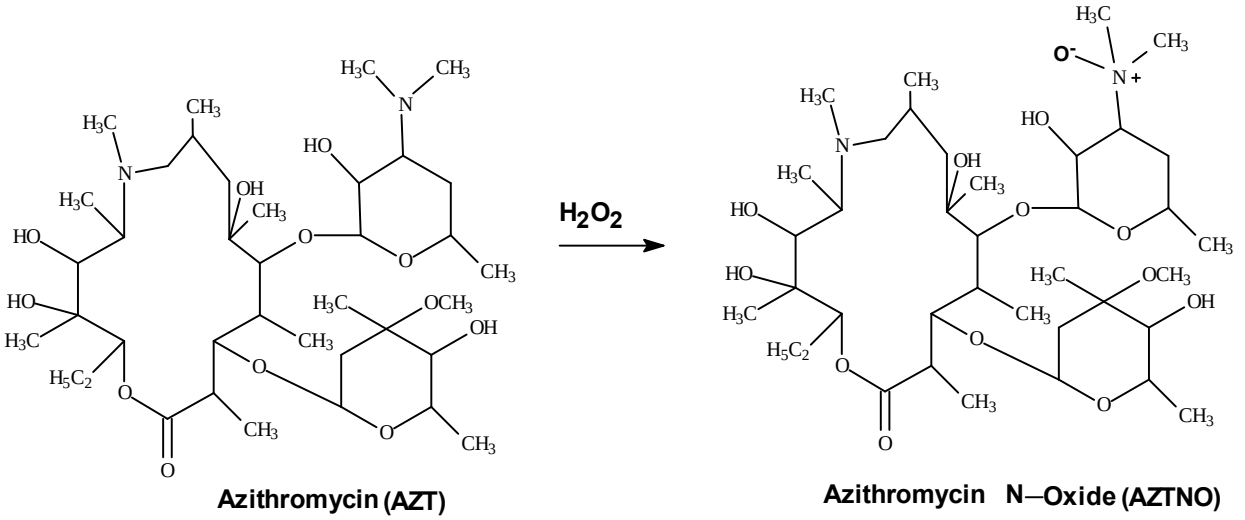


Figure 3.4: Oxidative degradation pathway of AZT.

To determine the order of reaction, the data was fitted into zero, first and second order kinetic models. The r^2 value of the linear plots was used as a measure of goodness of fit. The data generated (Table 3.7) fitted into the second order rate equation with linear regression equation $y=0.0106x+0.1377$ and $r^2=0.994$ (Figure 3.5). The half life of azithromycin in such oxidative environment was about 13 min. The rate constant (k) for this reaction was 1.06×10^{-2} area units^{-1}.min^{-1}.

This oxidative study may be indicative of the possible nature of degradation of AZT in the presence of oxidants. Examples of oxidants include reactive oxygen species such as hydrogen peroxide (H_2O_2), hypochlorous acid (HClO) and free radicals such as the hydroxyl radical ($OH^{\cdot}$) and the superoxide anion ($O_2^{\bar{1}}$).

Table 3.7: Oxidative degradation of azithromycin at 37 °C

Time (min)	Peak area	% Degradation
0	726457	0
10	652924	10.12
30	300637	58.62
60	175832	75.80
150	105842	85.43
210	44984	93.81
330	26656	96.33
390	23688	96.74
420	21296	97.07

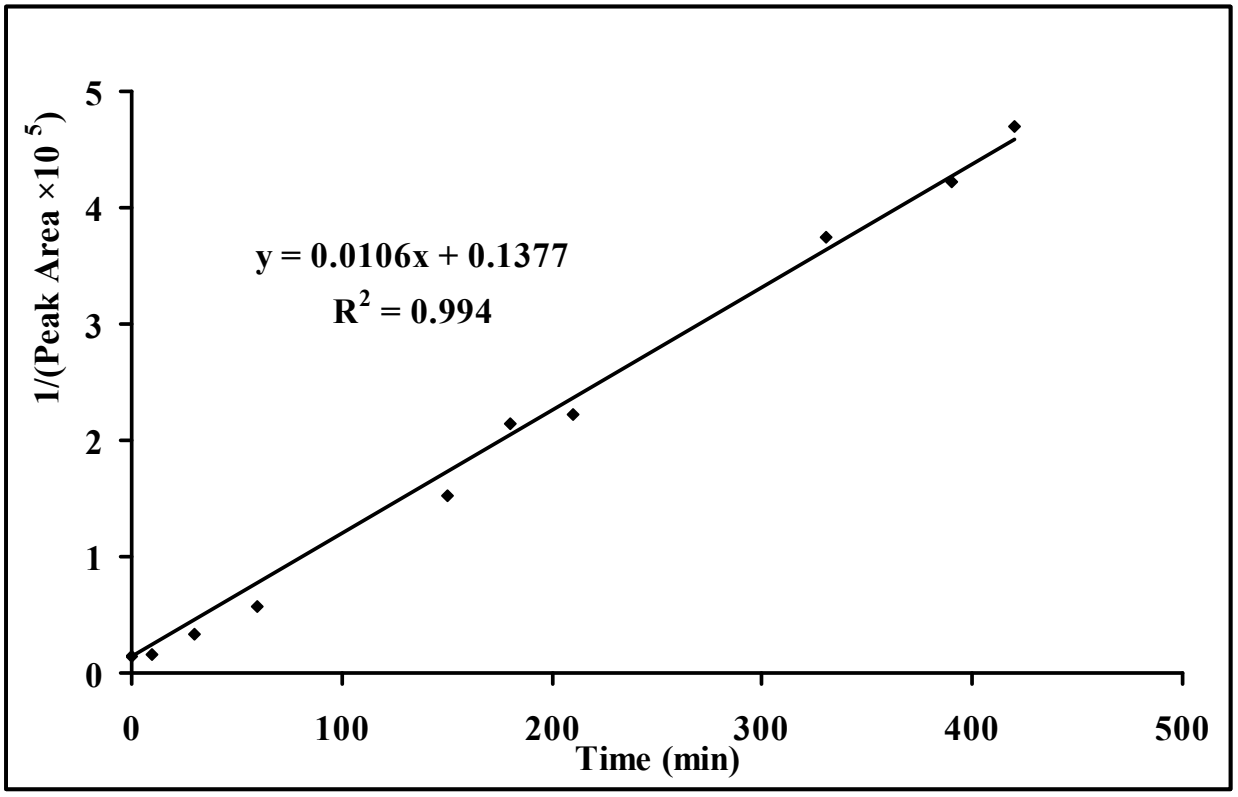


Figure 3.5: Degradation profile of AZT in 0.0005% v/v H_2O_2 at 37 °C.

Column: Waters XTerra® 5 µm C18, 250×4.6mm ID. Mobile Phase: Acetonitrile-0.1M Potassium phosphate pH 6.5-0.1M Tertbutylammonium hydroxide-Water (25:15:1:59, % v/v/v/v). Column temperature: 43 °C. Flow rate: 1.0 ml/min. Detection: 215 nm. Injection volume: 20 µl.

The AZT N-oxide formed as a result of oxidative degradation did not co-elute with any other eluted peak. Figures 3.6 and 3.7 indicate that the method can be employed to determine the content of AZT in the presence of oxidative degradation products and as such the proposed method is stability indicating.

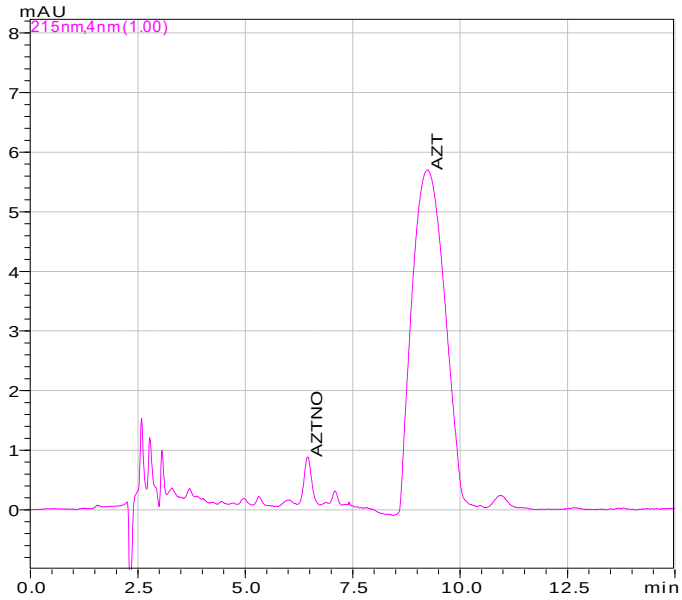


Figure 3.6: Typical chromatogram obtained after subjecting azithromycin to 0.0005 %v/v H_2O_2 at 37 °C for 30 min.

Column: Waters XTerra® 5 µm C18, 250×4.6mm ID. Mobile Phase: Acetonitrile-0.1M Potassium phosphate pH 6.5-0.1M Tertbutylammonium hydroxide-Water (25:15:1:59, % v/v/v/v). Column temperature: 43 °C. Flow rate: 1.0 ml/min. Detection: 215 nm. Injection volume: 20 µl.

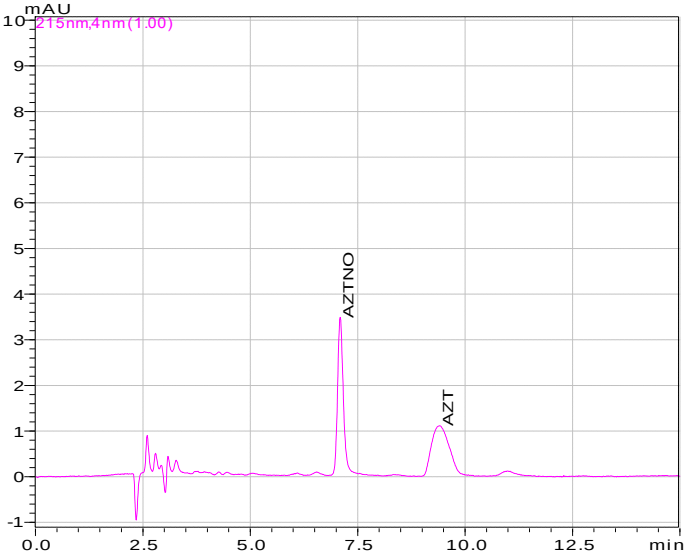


Figure 3.7: Typical chromatogram obtained after subjecting azithromycin to 0.0005 %v/v H_2O_2 at 37 °C for 6.5 h.

Column: Waters XTerra® 5 µm C18, 250×4.6mm ID. Mobile Phase: Acetonitrile-0.1M Potassium phosphate pH 6.5-0.1M Tertbutylammonium hydroxide-Water (25:15:1:59, % v/v/v/v). Column temperature: 43 °C. Flow rate: 1.0 ml/min. Detection: 215 nm. Injection volume: 20 µl.

3.8.2 Acid degradation of azithromycin

The presence of Ŭ-glycosidic bond linking the deoxy sugar (cladinose) to the macrocyclic lactone ring of azithromycin provides the basis for conducting acid degradation studies. Acid hydrolysis of the ether bond at position C3 (Figure 3.8) is the main decomposition pathway for AZT [32]. Degradation of azithromycin in gastric juice affects its bioavailability and consequently the therapeutic outcomes in patients.

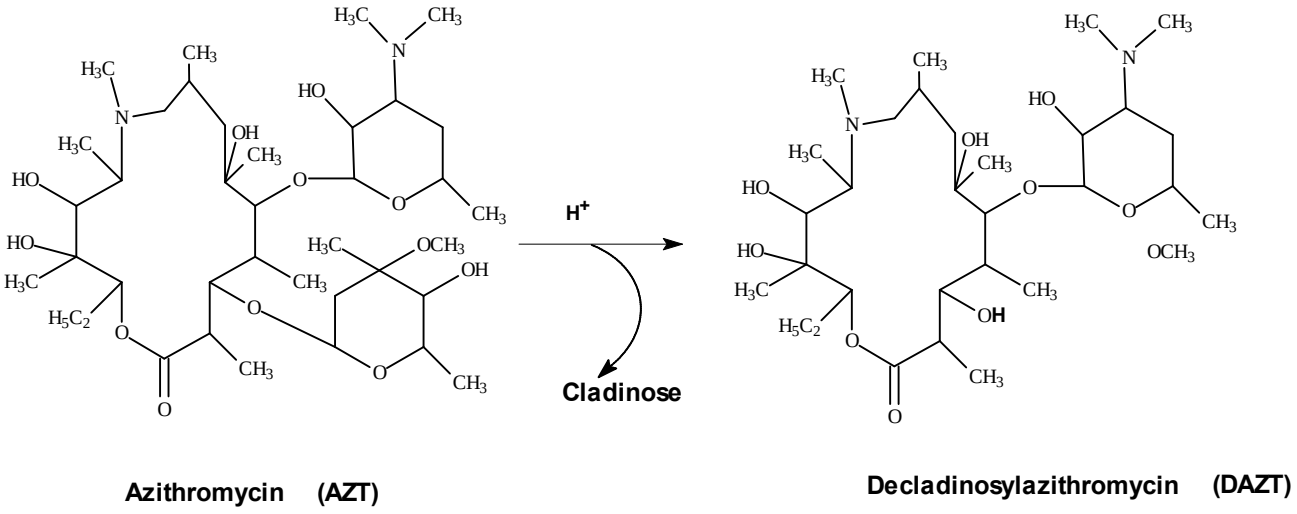


Figure 3.8: Degradation pathway for AZT in acidic environment

Acid degradation was carried out by incubating a 5 mg/ml solution of AZT in 0.1M (pH 1.60), 0.05M (pH 1.68), 0.025M (pH 1.92) and 0.01M H_3PO_4 (pH 2.37), respectively, at 37 °C using the method previously described by Abuga *et al*. [92]. The peak area of AZT was monitored using the developed optimized conditions. The peak areas were recorded as shown in Table 3.8.

The data generated (Table 3.8) showed that AZT degrades rapidly in strong acidic conditions with AZT base being degraded by about 90% when subjected to 0.1M and 0.05M H_3PO_4 at 37 °C within 10 min. Acid degradation at 0.01M H_3PO_4 (pH 2.37) was found to be too slow.

Therefore an intermediate concentration of acid 0.025M was used to carry out kinetics of decomposition.

The gastric emptying time of solutions ranges between 10-50 min [93, 94]. The range of the pH of gastric acid is 1.5-3.5 while its molarity may be as high as 0.1M [95, 96]. For instance, with a mean gastric residence time for AZT base as low as 10 min in gastric fluid of pH 1.60, 93% of the administered dose could be lost due to degradation in the stomach. The high proportion of drug that is degraded may impact negatively on the oral bioavailability of AZT.

Table 3.8: Acid degradation of azithromycin in 0.1M and 0.05M H_3PO_4 at 37 °C

Time (min)	Peak area	
	0.1M H_3PO_4	**0.05M H_3PO_4**
0	726457 (0)	727981 (0)
5	53872 (92.6)	87104 (88.0)
10	46450 (93.6)	57469 (93.1)

The figures in parenthesis represent percent degradation.

The order of reaction was determined by plotting ln peak area (first order) and reciprocal peak area (second order) versus time graphs. The data generated (Table 3.9) fitted into the first order rate equation with linear regression equation y= -0.0002x +13.651 and r^2=0.9907 as shown in Figure 3.9. The half life of azithromycin in such acidic environment is about 3465 min (57.8 h). The rate constant (k) for this reaction was 2.0 $\times 10^{-4}$ min^{-1}. In all instances, the degradation products in acid were well separated from the main component, a further evidence of the stability indicating ability of the method.

Table 3.9: Acid degradation of azithromycin in 0.025M H_3PO_4 at 37 °C

Time (min)	Peak area	% Degradation
0	813143	0
701	733193	9.8
793	718604	11.6
854	717227	11.8
977	714903	12.1
1007	710407	12.6
1128	705436	13.2
1189	700790	13.8
1311	680785	16.3
1372	673272	17.2
1402	672661	17.3
1433	670516	17.5
2886	513587	36.8
2908	501785	38.3
3924	413336	49.2
4173	389459	52.1
4484	327061	59.8
5314	250754	69.2

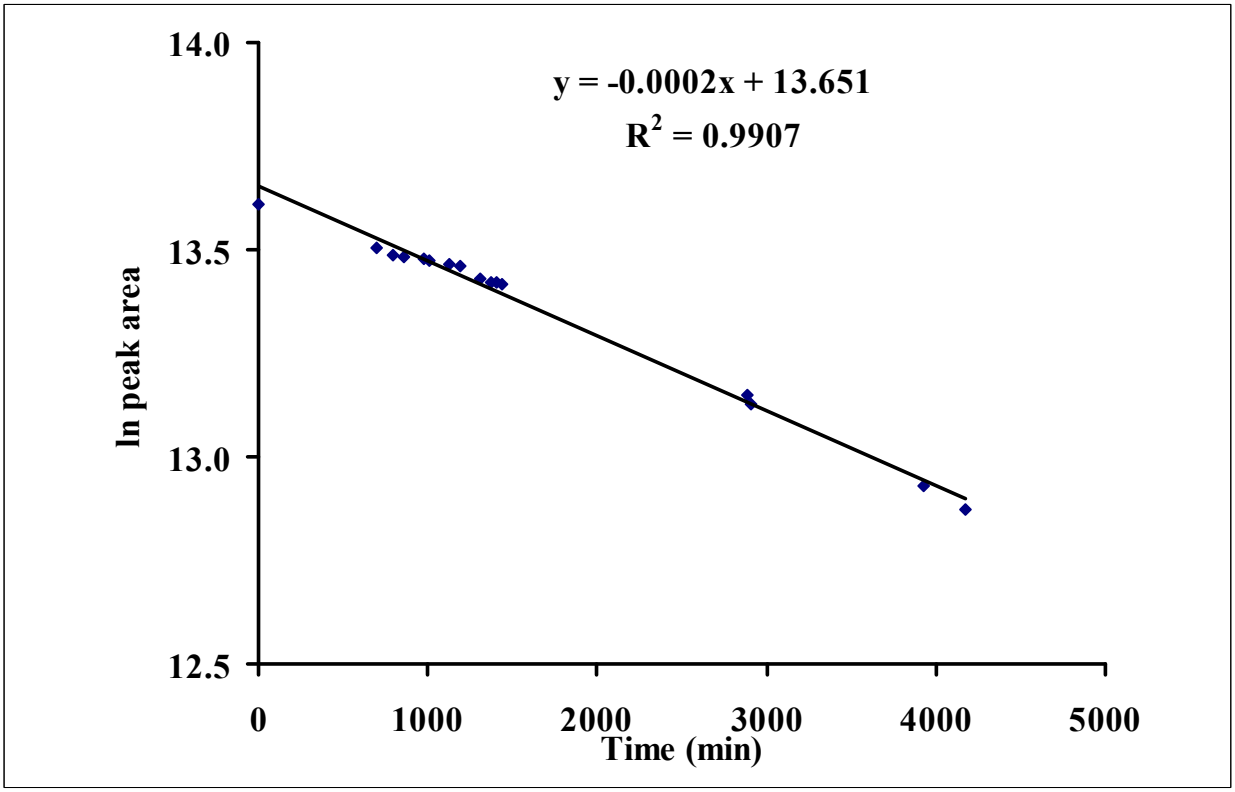


Figure 3.9: Acid degradation profile of AZT in 0.025M H_3PO_4 at 37 °C.

Column: Waters XTerra® 5 µm C18, 250×4.6mm ID. Mobile Phase: Acetonitrile-0.1 Potassium phosphate pH 6.5-0.1M Tertbutylammonium hydroxide-Water (25:15:1:59, % v/v/v/v). Column temperature: 43 °C. Flow rate: 1.0 ml/min. Detection: 215 nm. Injection volume: 20 µl.

3.9 Stability of the azithromycin in diluent

The stability of azithromycin was monitored using the developed method. This was done by preparing a 5 mg/ml azithromycin working standard and monitoring the peak areas of azithromycin on day 1, 2, 3 and 7. The sample was kept at in room maintained at 20° C. The peak areas are recorded in Table 3.10. This showed that at room temperature, AZT is degraded by about 6% when dissolved in acetonitrile-water (50:50) for one week.

Table 3.10: Degradation of AZT in diluent

Day	Peak area	% Degradation
1	814416	0
2	791527	2.8
3	784882	3.6
7	767861	5.7

Column: Waters XTerra® 5 µm C18, 250×4.6mm ID. Mobile Phase: Acetonitrile-0.1M Potassium phosphate pH 6.5-0.1M Tertbutylammonium hydroxide-Water (25:15:1:59, % v/v/v/v). Column temperature: 43 °C. Flow rate: 1.0 ml/min. Detection: 215 nm. Injection volume: 20 µl.

CHAPTER FOUR

ANALYSIS OF SAMPLES

4.1 Introduction

The method developed in this study was intended for use in evaluating the quality of azithromycin tablets and suspensions marketed in Kenya. The reliability of the method for its intended use was tested by using it to evaluate the quality of commercially marketed tablet and suspension formulations.

4.2 Acquisition of samples

4.2.1 Registered azithromycin formulations

The online drug register maintained by the Pharmacy and Poisons Board (PPB) listed 77 azithromycin containing registered products that were marketed in Kenya [97] as summarised in Table 4.1.

Table 4.1: Summary of the registered azithromycin formulations

Formulation	Dosage strength	No. of products
Suspension	40 mg/5 ml	21 (27.27)
Tablet	250 mg	9 (11.69)
Tablet	500 mg	31 (40.26)
Capsule	250 mg	6 (7.79)
Capsule	500 mg	4 (5.19)
Injection	500 mg	3 (3.90)
Combination	1000 mg	3 (3.90)
Total		**77 (100)**

Figures in parentheses represent the relative proportions of the formulation expressed as %

The combination AZT products were those that were azithromycin co-packed with other antimicrobial agents such as fluconazole and secnidazole.

It was also noted that there are only two brand of azithromycin that are manufactured locally representing 2.6% of the azithromycin-market. This calls for the government to formulate and implement policies that are geared towards encouraging local production of such products.

It was also observed that two brands of the sampled products were not appearing on the online register. This may be due to delayed updating of the online PPB drug-register or that the products were not registered by PPB. It is imperative that the PPB promptly posts the list of registered products on their website for reference by healthcare professionals, relevant organizations and even patients.

4.2.2 Sampling

Azithromycin 500 mg tablets and suspensions accounted for 31% and 21%, respectively, of the azithromycin registered products in the market. These two formulations were selected for analysis since they also represent the standard dosage forms for adults and paediatrics respectively.

The test samples were purchased from randomly selected retail pharmacies located within the Central Business District of the city of Nairobi. For the suspensions a sample of nine was used with five of the suspensions having been premixed while 4 were not. Out of the four AZT powder for oral suspensions, one was the innovator brand. On the other hand, thirteen tablet brands of azithromycin were sampled for analysis with one of them being the innovator brand.

Azithromycin raw material was a donation from SkyLight Chemicals, Industrial Area, Nairobi while Zithromax® tablets and suspensions were kind donations by Pfizer Laboratories, Industrial Area, Nairobi. The registration status of Zithromax® tablets and suspensions was confirmed with the PPB list.

Table 4.2: Summary of products sampled from the market

Product Code	Formulation Type	Product listed on the PPB online register	Batch number	Date of manufacture	Expiry date
I	Tablet	Y	FAA3219C	11/2012	10/2014
	Tablet	Y	FAA3219E	11/2012	10/2014
II	Tablet	Y	L017	08/2012	08/2015
	Tablet	Y	L019	08/2012	08/2015
III	Tablet	Y	R62021	10/2012	09/2014
	Tablet	Y	R62019	10/2012	09/2014
IV	Tablet	Y	333P302X	03/2013	02/2016
	Tablet	Y	333P301X	03/2013	02/2016
V	Tablet	Y	320054	02/2013	01/2016
VI	Tablet	Y	ABV1A34	01/2013	12/2014
	Tablet	Y	ABV1A24	01/2013	12/2014
VII	Tablet	Y	ATE0306	07/2012	06/2015
	Tablet	Y	ATE0308	07/2012	06/2015
VIII	Suspension (M)	Y	L017	09/2012	09/2014
	Suspension (M)	Y	L014	07/2012	07/2014
IX	Suspension (M)	Y	3549203X	07/2012	06/2015
	Suspension (M)	Y	3549204X	07/2012	06/2015
X	Suspension (M)	Y	FAA8310D	04/2013	03/2015
XI	Suspension (M)	Y	ABT1A33	01/2013	12/2014
XII	Tablet	Y	AIBHK2007	12/2012	11/2015

XIII	Tablet	Y	TN1007	03/2011	03/2014
XIV	Tablet	N	AB-1301	01/2013	12/2015
XV	Tablet	Y	MK-14	03/2013	02/2015
XVI	Tablet	N	TE-4195	12/2012	11/2015
XVII	Suspension (P)	Y	WOP3001	01/2013	12/2014
XVIII	Suspension (P)	Y	DG2005	10/2012	10/2014
XIX	Suspension (M)	Y	ALE0022	08/2012	07/2014
XX	Suspension (P)	Y	121005B	10/2012	10/2015
Raw material	Powder	N/A	_	07/2012	06/2016
Zithromax®	Suspension (P)	Y	206502	03/2012	03/2015
Zithromax®	Tablet	Y	218018	06/2012	06/2014

P=Powder for reconstitution, M=Premixed suspension, N=No, Y=Yes, N/A=Not applicable,

4.3 Analysis of samples

One batch of each product, where possible two batches, was sampled for analysis using the developed HPLC method.

4.3.1 Preparation of samples for analysis

4.3.1.1 Standard solution

Azithromycin working standard, 25 mg, was weighed and transferred to a 5 ml volumetric flask, dissolved in 2.5 ml of acetonitrile and the solution sonicated for 15 min. The solution was then made up to volume with distilled water and injected onto the column.

4.3.1.2 Sample solutions

The USP (2012) sample preparation method [55] was adopted both for the analysis of sampled tablets and suspensions.

In the analysis of tablets, twenty tablets were weighed and pulverized using a mortar and pestle and tablet powder equivalent to 250 mg azithromycin was transferred into a 50 ml volumetric flask followed by addition of 25 ml acetonitrile. The resultant solution was sonicated for 15 min, filled to the mark with distilled water and filtered through a 0.45 µm membrane filter. The filtrate was then chromatographed.

The relative density of the suspension samples was determined upon reconstitution, where applicable, and suspension equivalent to 40 mg/ml weighed in 5 ml volumetric flasks. This was followed by addition of 2.5 ml of acetonitrile. The resultant solution was sonicated for 15 min, filled to the mark with distilled water and filtered through a 0.45 µm membrane filter. The filtrate was then chromatographed. Azithromycin raw material was prepared using the procedure described for the standard solution in the USP (2012).

The test and standard solutions were run with triplicate under the optimum HPLC conditions. The peak areas obtained were used for the determination of the content of AZT in the samples.

4.3.2 Assay results

Thirteen of the analysed products were tablets while nine were suspensions of azithromycin. Out of the nine suspensions sampled, five were the premixed suspensions and the rest were dry powders for reconstitution.

The assay results (Table 4.3) were evaluated for assay specification of the product. The results obtained revealed that three (23%) tablet brands (n=13) did not meet the USP [55] specifications for assay (90.0-110.0 % label claim). Three (60%) premixed suspensions (n=5) and one (25%)

dry powder for reconstitution (n=4) failed to comply with the USP (2012) specifications for assay as shown in Table 4.3.

Table 4.3: Assay values of selected marketed samples of azithromycin

Product Code	Formulation Type	Content Found (%)	Remarks
I	Tablet	93.6 (1.55)	C
	Tablet	94.8 (1.04)	C
II	Tablet	110.3(0.16)	C
	Tablet	109.1(0.12)	C
III	Tablet	98.5 (0.46	C
	Tablet	99.2 (0.31)	C
IV	Tablet	97.2 (0.19)	C
	Tablet	97.6 (0.19)	C
V	Tablet	102.0 (1.51)	C
VI	Tablet	109.9 (0.32)	C
	Tablet	107.3 (0.26)	C
VII	Tablet	105.1 (1.60)	C
	Tablet	104.3 (1.28)	C
VIII	Suspension (M)	110.0 (0.61)	C
	Suspension (M)	109.3 (0.47)	C
IX	Suspension (M)	96.4 (0.72	C
	Suspension (M)	96.1 (0.18)	C
X	Suspension (M)	69.3 (1.68)	NC
XI	Suspension (M)	89.3 (0.39)	NC
XII	Tablet	81.4 (0.46)	NC
XIII	Tablet	99.8 (0.31)	C
XIV	Tablet	83.3 (0.15)	NC
XV	Tablet	87.0 (1.13)	NC
XVI	Tablet	103.3 (1.33)	C
XVII	Suspension (P)	88.6 (0.16)	NC

XVIII	Suspension (P)	105.5 (0.36)	C
XIX	Suspension (M)	87.8 (1.32)	NC
XX	Suspension (P)	98.7 (0.66)	C
Raw material	Powder	94.7 (0.09)	C
Zithromax®	Suspension (P)	99.6 (0.71)	C
Zithromax®	Tablet	96.4 (1.48)	C

The figures in parentheses represent the CV, P=Powder for reconstitution, M=Premixed NC=Did not comply, C=Complied.

Figures 4.1-4.7 show typical chromatograms obtained during the assay of samples of azithromycin.

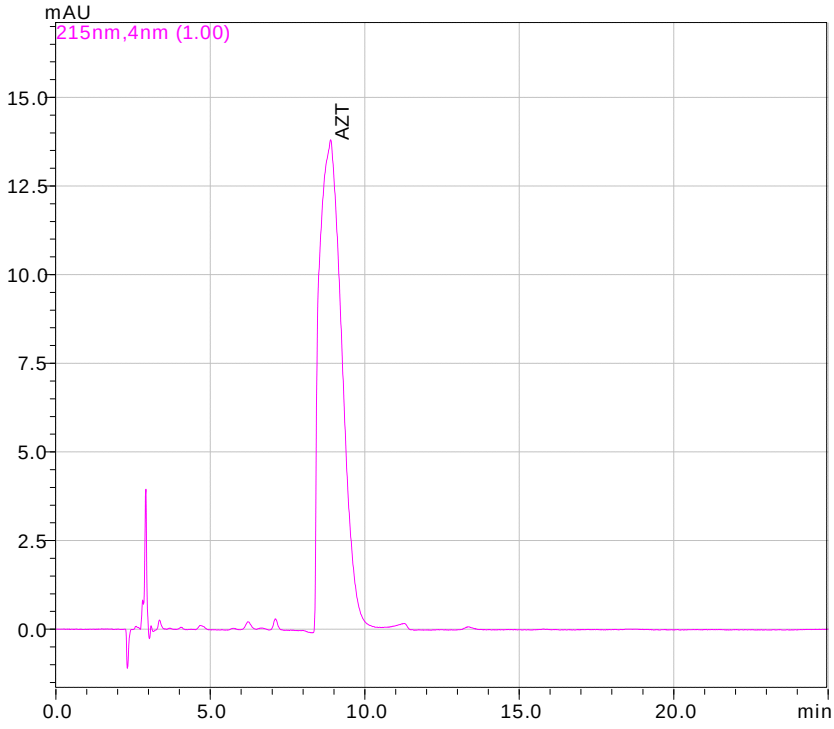


Figure 4.1: Typical chromatogram of azithromycin standard.

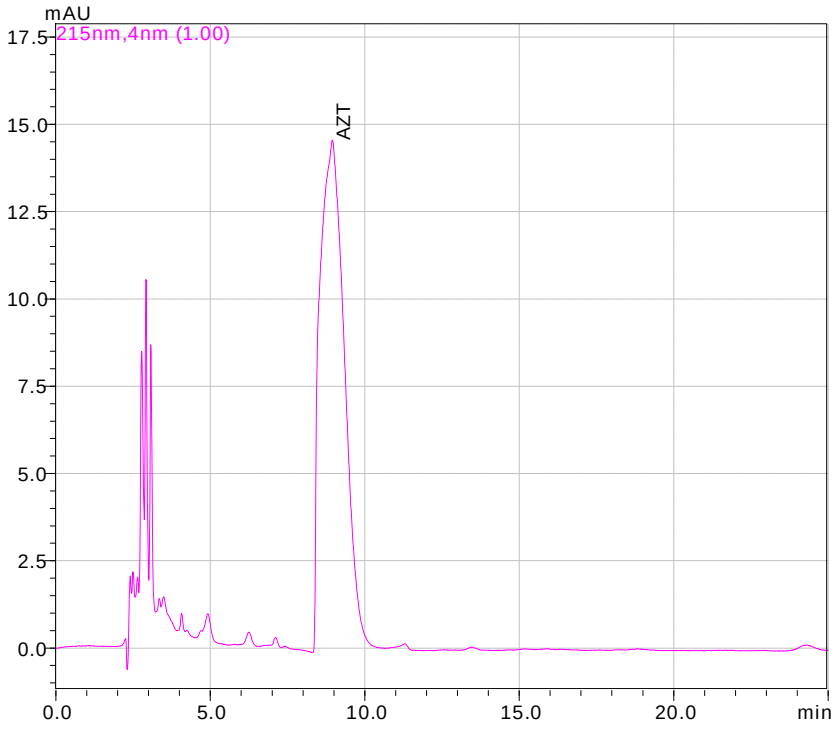


Figure 4.2: Typical chromatogram of sample I.

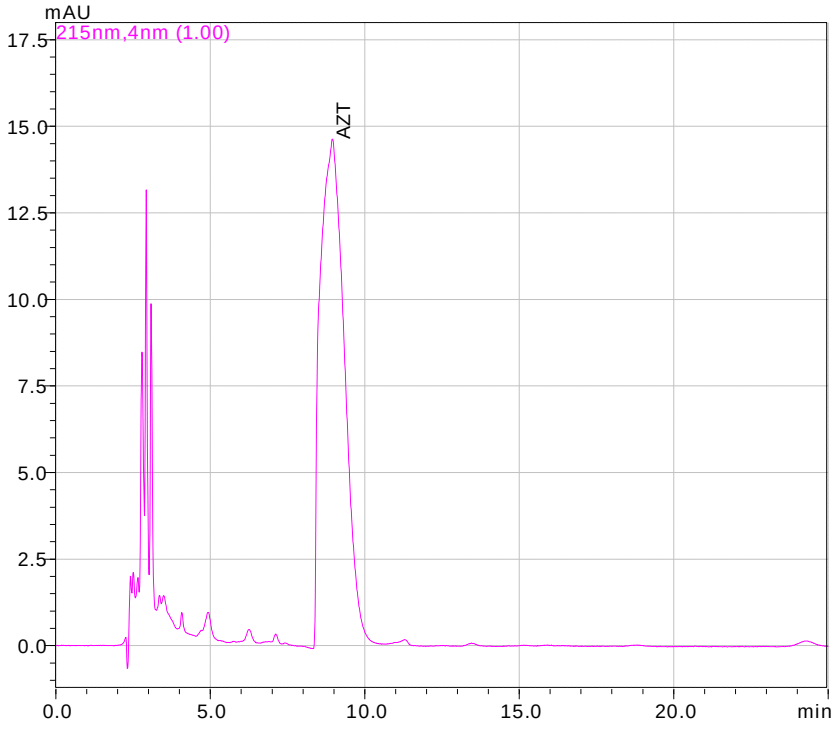


Figure 4.3: Typical chromatogram of sample II.

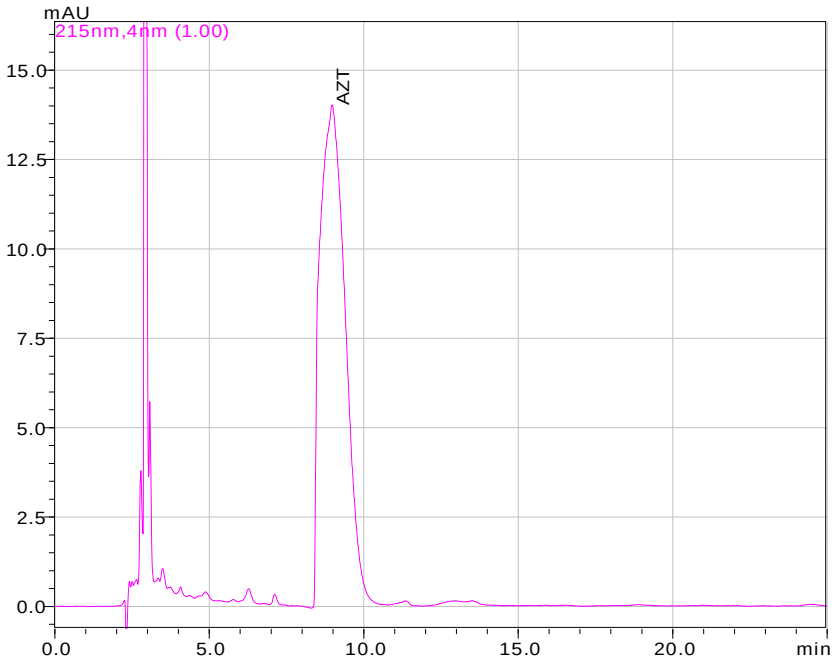


Figure 4.4: Typical chromatogram of sample VI.

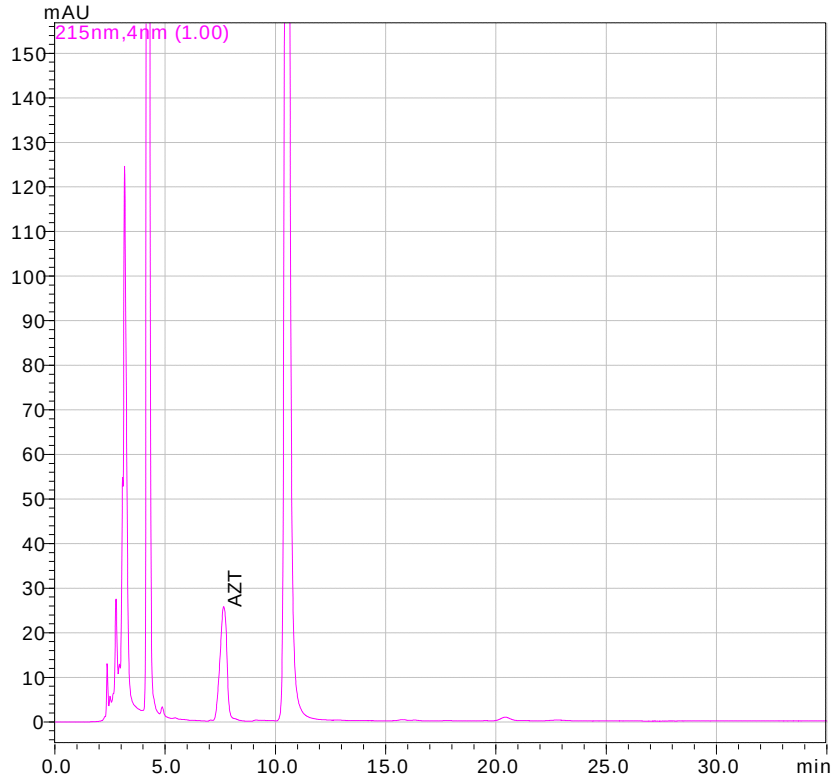


Figure 4.5: Typical chromatogram of sample VIII.

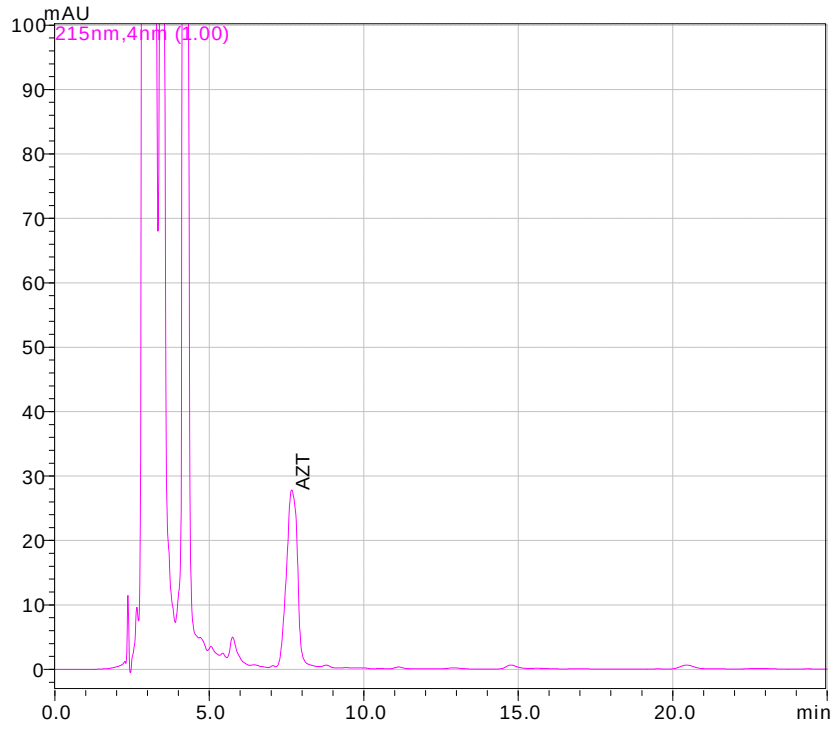
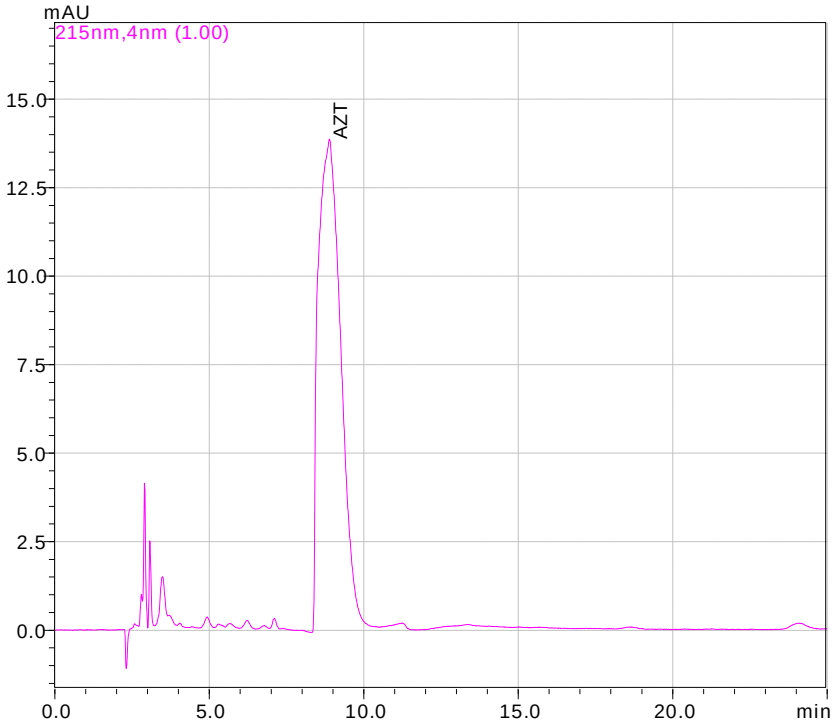


Figure 4.6: Typical chromatogram of sample XI.



4.7: Typical chromatogram of azithromycin raw material.

The assay results (Table 4.3) also indicated that the content for premixed suspensions was relatively lower compared to that of dry powders for reconstitution. This suggests the nature of formulation impacts on the content of the active pharmaceutical ingredient. The low content found in the premixed suspensions may be due to instability of azithromycin in solution as a result of decladinosylation that may occur when the product is in solution. Decladinosylation in such premixed suspensions is time-dependent which means that the AZT content decreases during the shelf life of such products. It is noteworthy that the innovator suspension is a dry powder for reconstitution. These findings underscore the need for the manufacturer to carry out comprehensive studies to justify any formulational aspects that significantly differ from that of the innovator products. The Drug Regulatory Authority (DRA) ought to be vigilant on products that deviate from the innovator formulation. This is to ensure that generics registered in the country are pharmaceutically equivalent with the innovator brand. Where significant changes in formulation occur, the generic manufacturer should provide sufficient data to support the rationale for such deviation.

One of three tablet brands that did not meet the assay specifications appeared in the online register of the PPB. This underscores the need by the regulatory authority to carry out continuous market surveillance to monitor the products available in the market and their quality performance. This will help curb the emergence of macrolide-resistant strains thus preserving the antibiotic arsenal to treat susceptible microorganisms.

4.3.3 Limitations of the sample analysis

It was not possible to obtain more than one batch of the same formulation except for a few products since there is a sole country agent for each brand in the market. Therefore it was impossible to evaluate batch to batch variation among products. However this may be achieved by extending the sampling period by 6-12 months to allow for new batches to reach the market. It was also difficult to get products from some pharmacies due to suspicion by the sellers.

CHAPTER FIVE

GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 General discussion

The melting of azithromycin crystals is preceded by the loss of water. Azithromycin dihydrate had a melting range of 134.7-141.4 ºC. The melting point for the monohydrate form was not determined. The melting value obtained of the dihydrate form was consistent with the values reported in literature. A thermal differential calorimetric scan would have shown how the loss of water occurs and the transition from the dihydrate to the monohydrate and eventually to the anhydrous azithromycin.

Quantitative determination of impurities by any analytical method is highly desired. The ICH specifies that impurities occurring in new drug substances in <0.1% should be structurally characterized. The limits for identified impurities are set in reference to the safety data profiles while any unidentified impurities should not exceed the 0.1% level. The developed liquid chromatographic method was able to separate and quantify impurities up to the 0.1% level as indicated by the LOQ. The unknown impurities, I-III, were present above the disregard limit and therefore further research should be done to characterize them as specified by ICH [88].

Phosphate buffers produce more stable analytical conditions, in addition to providing a buffering capacity over a wide range of pH as a result of having three pKa values for the acid, H_3PO_4, pKa = 2.21 and phosphate ions, $H_2PO_4^-$, pKa= 7.21; and HPO_4^{2-}, pKa = 12.67 [98]. These pKa values

are sufficiently different so that at any one pH only the members of a single conjugate pair are present in significant concentrations. The UV transparency of phosphate buffers Ò190 nm [98] and therefore they are preferred in low wavelength UV experiments. The use of acetate buffers was ruled out due to their low column efficiency associated with them.

The use of ion pairing agents in the optimized method is associated with difficulties in cleaning the columns. It is recommended to use a cleaning method that uses the mobile phase minus the buffer and ion pair. For this case acetonitrile and water was used at 50:50 respectively. The difficulty in cleaning such columns is based on the fact that ion pairs partition well into the bonded phase of reverse phase columns. Cleaning is important since the sorbed materials may build up to levels high enough that they begin to act as an alternative stationary phase as analytes interact with these impurities contributing to the separation mechanisms. This results in tailing and shift of retention times. Too much contamination may results in increased column back pressures that may create voids in the column. Also dirty columns give baseline artefacts.

The developed liquid chromatographic method for the analysis of azithromycin in tablets, raw materials and suspension exhibited good linearity, precision, sensitivity, simplicity making it an alternative method for the analysis of AZT containing products in the market. The method demonstrated high specificity as it was used to determine AZT in the presence of related substances. Additionally, the method was found to be withstanding small deviations in the chromatographic conditions from the optimum. The method can be used to determine AZT in the presence of degradation products arising from stress testing in acidic or oxidative conditions. With a run time of 25 min, the method allows relatively faster analysis compared to others reported in literature for the determination of AZT and related substances.

The developed method was simple and less likely to cause baseline problems during routine use. The successful application of the method in the analysis of marketed products of azithromycin was further evidence of the utility of the method.

The finding of products that do not meet assay specifications in the market is a matter of public health concern and it underscores the need for sustained post market surveillance by the Drug Regulatory Authority (DRA). Also there is the need to curb counterfeits of innovator and generic brands through such surveillance. The present study could not unearth counterfeits due to the limited sampling done within the Central Business District of the city of Nairobi. It would require extensive countrywide sampling to fully investigate the prevalence of counterfeits in the Kenyan market. The innovator product was provided by the manufacturer and therefore it was not possible to unearth any counterfeiting of the product.

5.2 Recommendations

The unknown impurities that were present in the artificial working mixture should be characterized using LC-MS in order to improve the scope of the applicability of the method.

Collaborative studies between laboratories are required to assess the ruggedness of the proposed method. This will ultimately improve on the applicability of the method in other laboratory settings.

The linearity studies are required to demonstrate the applicability of the method in the determination of each of the impurities separated using the developed method.

There is also the need to carry out comparative dissolution profiles for the azithromycin tablets and suspensions since dissolution profiles differ among the brands of products and this imparts on the bioavailability and efficacy when administered to patients. The finding that about 90% of AZT base is degraded in 10 min, in acid with pH 1.6-1.7, is indicative of the acid-sensitivity of this compound. If not suitably formulated to guard against gastric acid, AZT base quickly degrades thus negatively affecting the oral bioavailability of such products. To ascertain therapeutic equivalence among azithromycin brands, bioequivalence studies are mandatory.

5.3 Conclusion

A simple, rapid, isocratic and sensitive liquid chromatographic method was developed for determination of AZT raw material, tablet and suspension dosage forms. The optimum chromatographic conditions were: Analytical column: Waters XTerra® C18, 250×4.6 mm and 5 µm particle size maintained at 43 °C. Mobile phase: Acetonitrile-0.1M KH_2PO_4 pH 6.5-0.1M tetrabutylammonium hydroxide-water (25:15:1:59, % v/v/v/v) delivered at a flow rate of 1.0 ml/min and UV detection at 215 nm. The developed method was validated and applied for the analysis of AZT raw material, tablet and suspension dosage formulations. The linearity equation and correlation coefficient for azithromycin were y=9948x-40457 and 0.997 respectively. The method was robust with the robustness range for pH being 6-7 pH units, 22-28% v/v for acetonitrile and 41-45 °C for temperature.

The method was used for carrying out market surveillance for azithromycin products in the Central Business District of the city of Nairobi. Assay results indicated that 23.1% (n=13) AZT tablets did not meet the USP (2012) specifications for assay while 44.4% (n=9) AZT suspensions did not.

The premixed suspensions accounted for about 33.3% of all the suspensions sampled that did not meet assay specifications. There is need for comprehensive comparative studies on the content of AZT in premixed suspensions and dry powders for reconstitution since a larger sample size would be more indicative of the quality differences between the two formulations.

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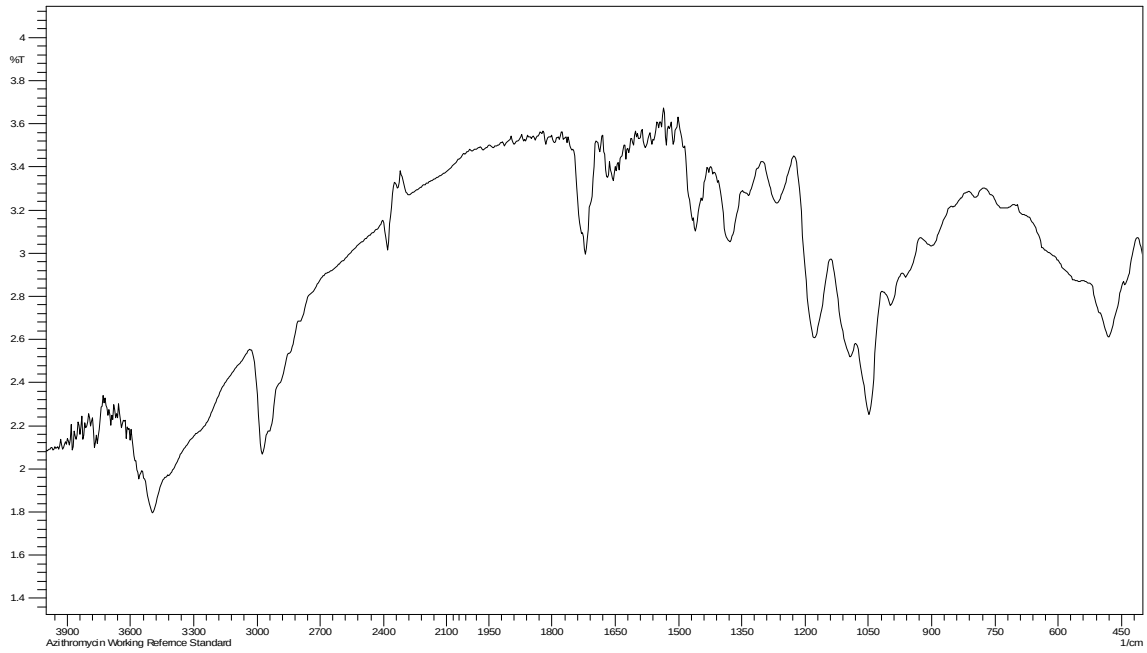
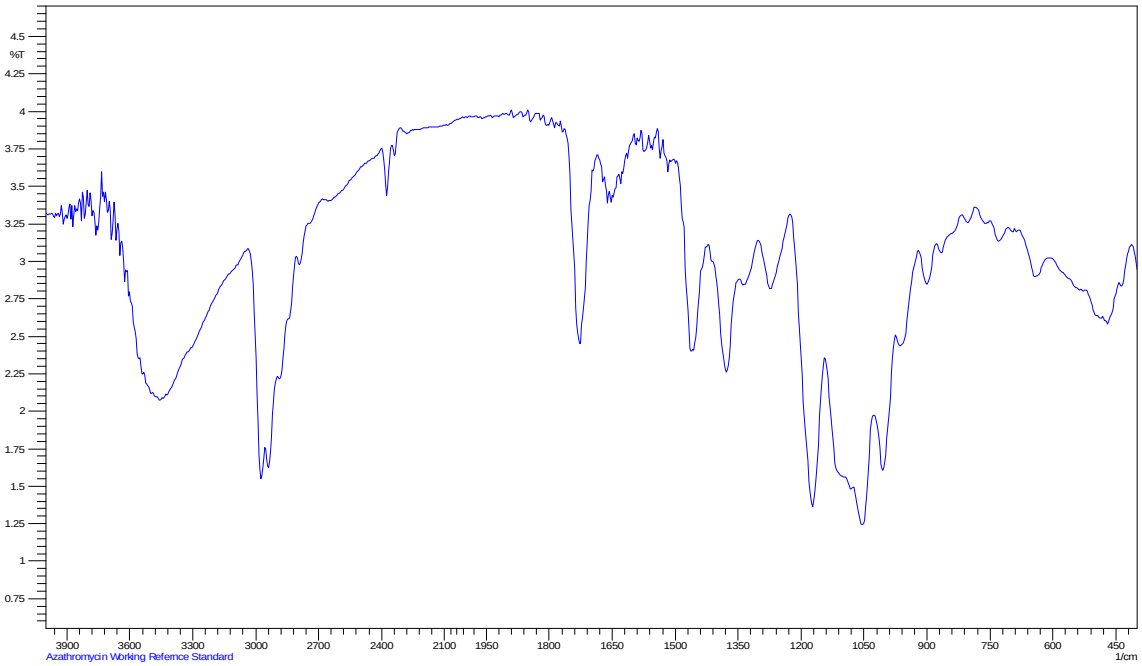
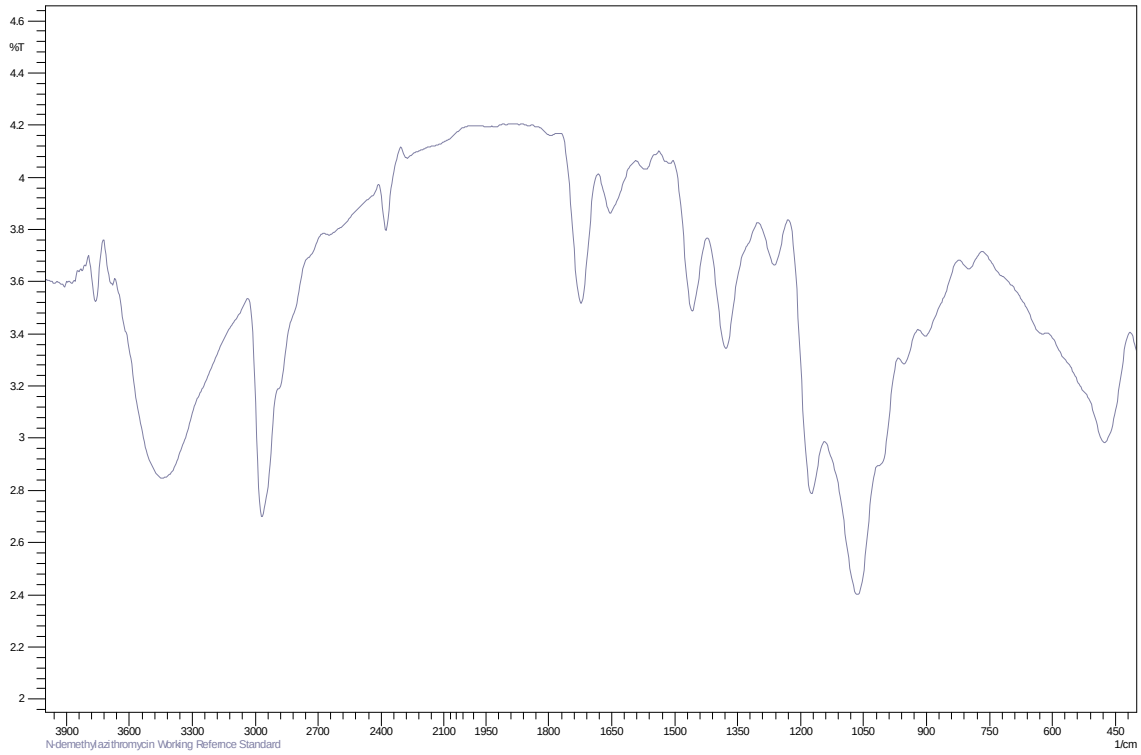
APPENDICES



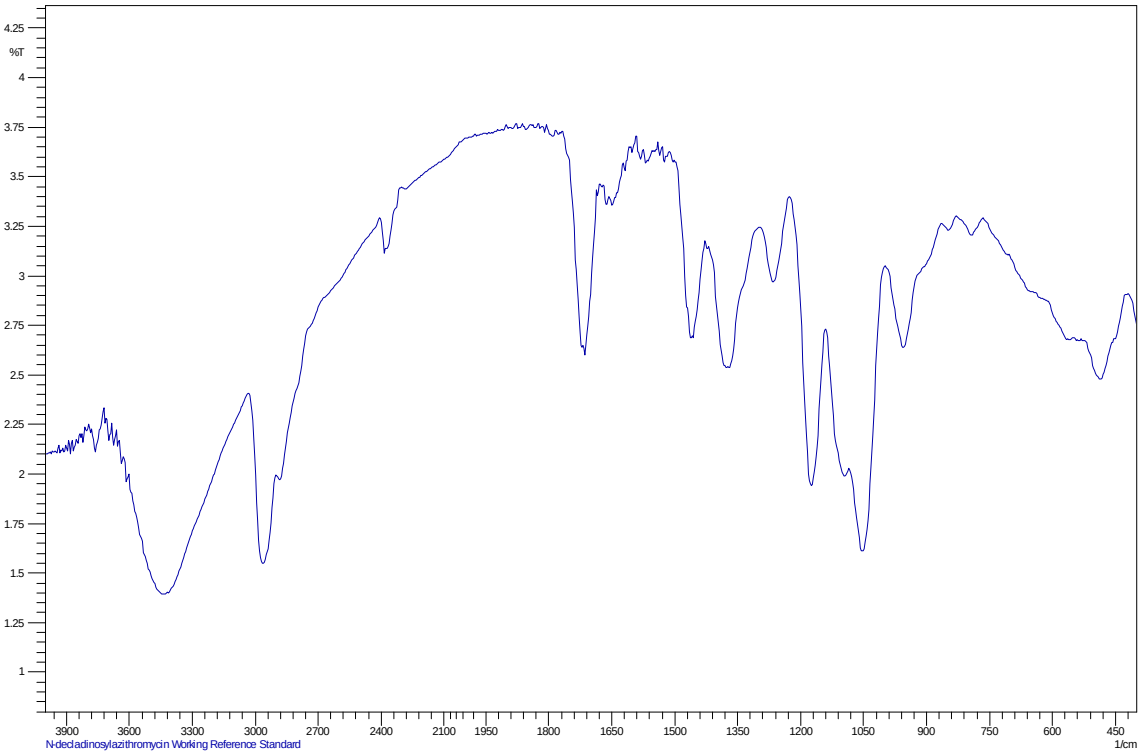
Appendix 1: FTIR spectrum of azithromycin (KBr disk)



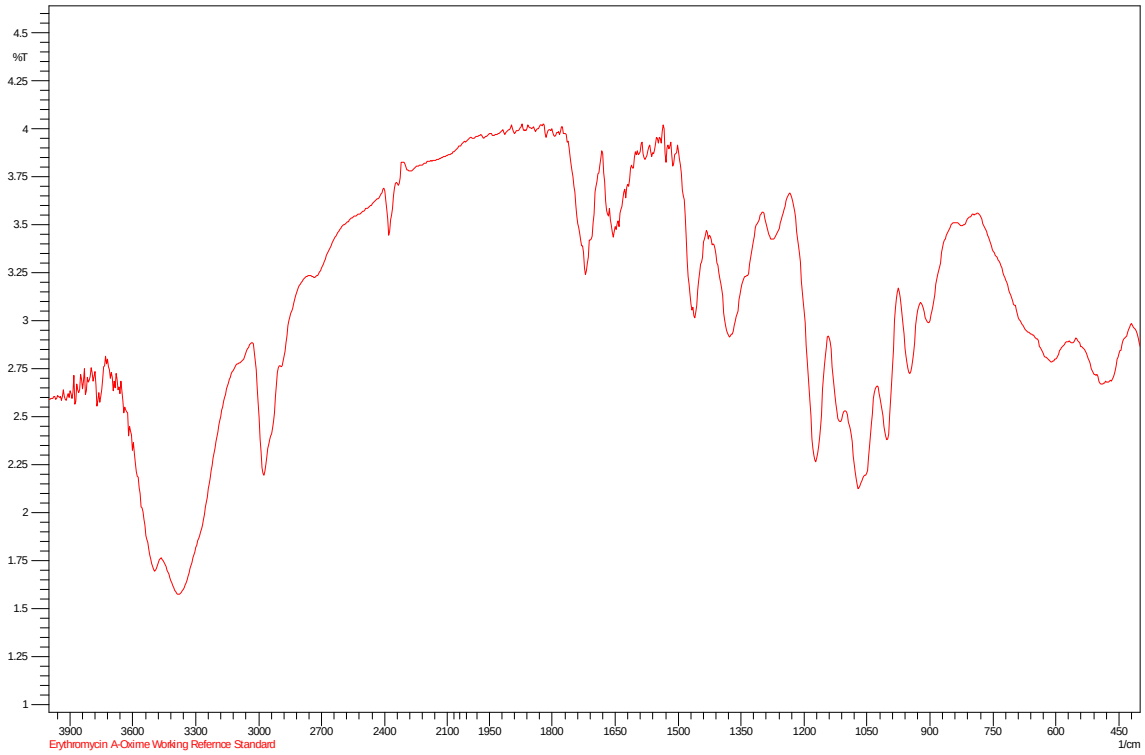
Appendix 2: FTIR spectrum of azathromycin (azaerythromycin) (KBr disk)



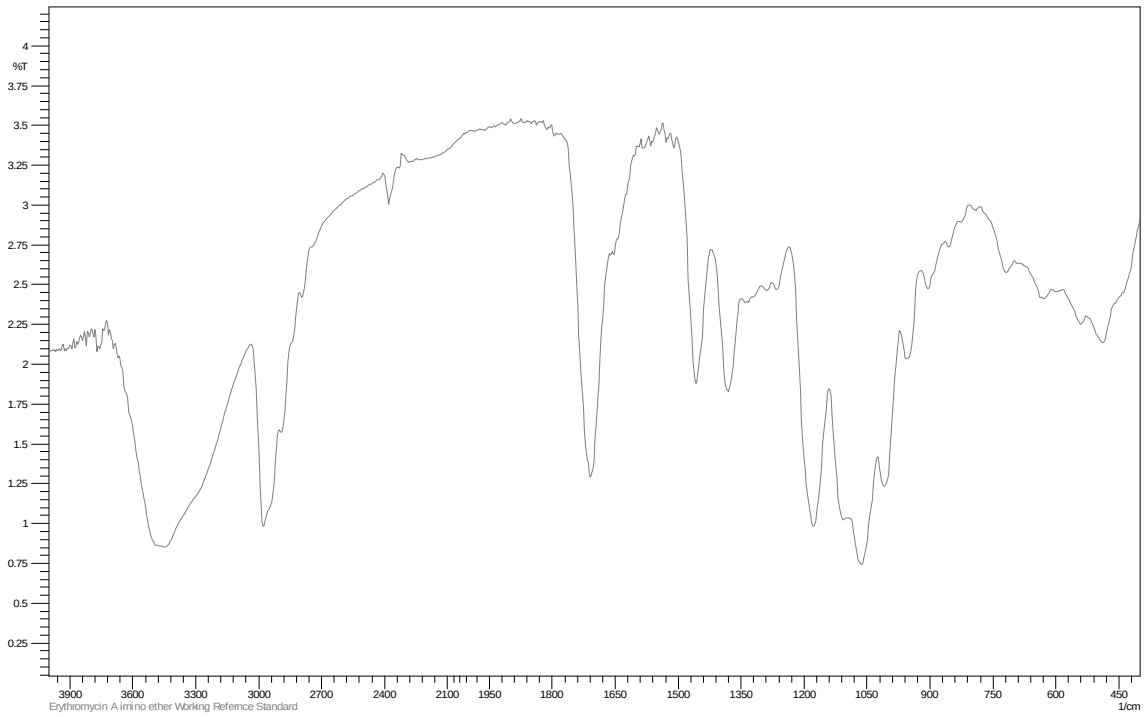
Appendix 3: FTIR spectrum of N-demethylazithromycin (KBr disk)



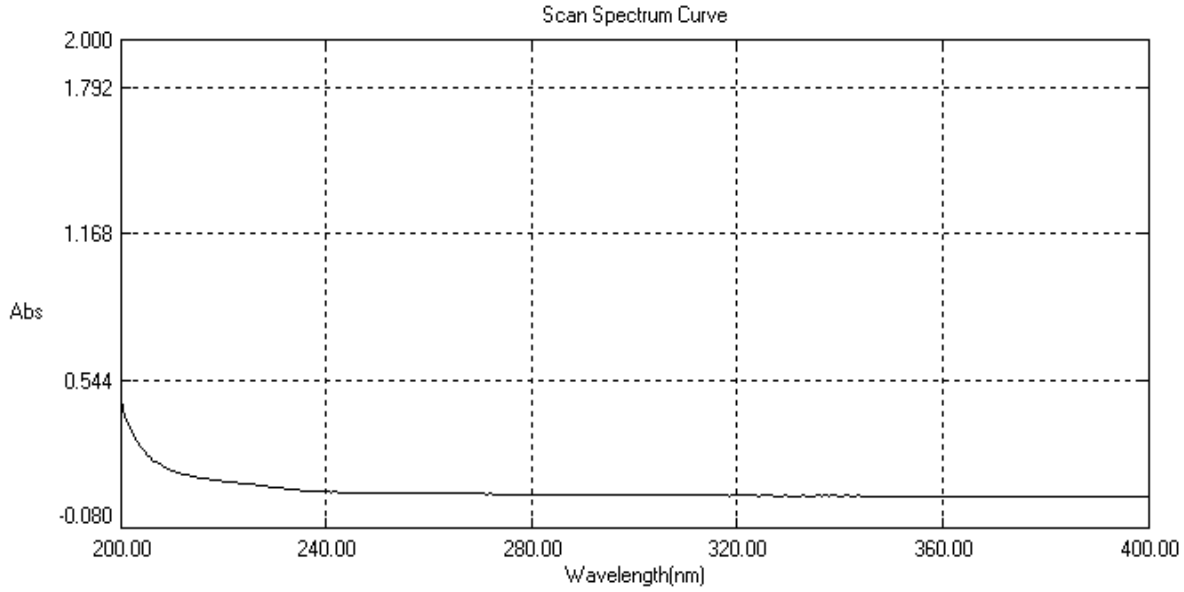
Appendix 4: FTIR spectrum of decladinosylazithromycin (KBr disk)



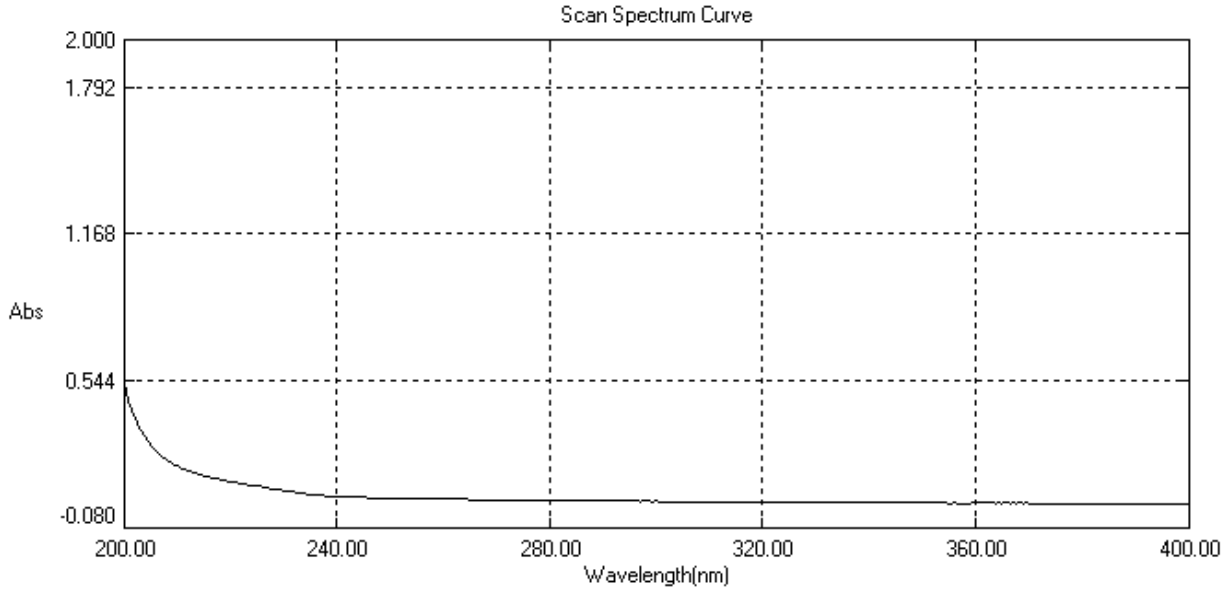
Appendix 5: FTIR spectrum of erythromycin A oxime (KBr disk)



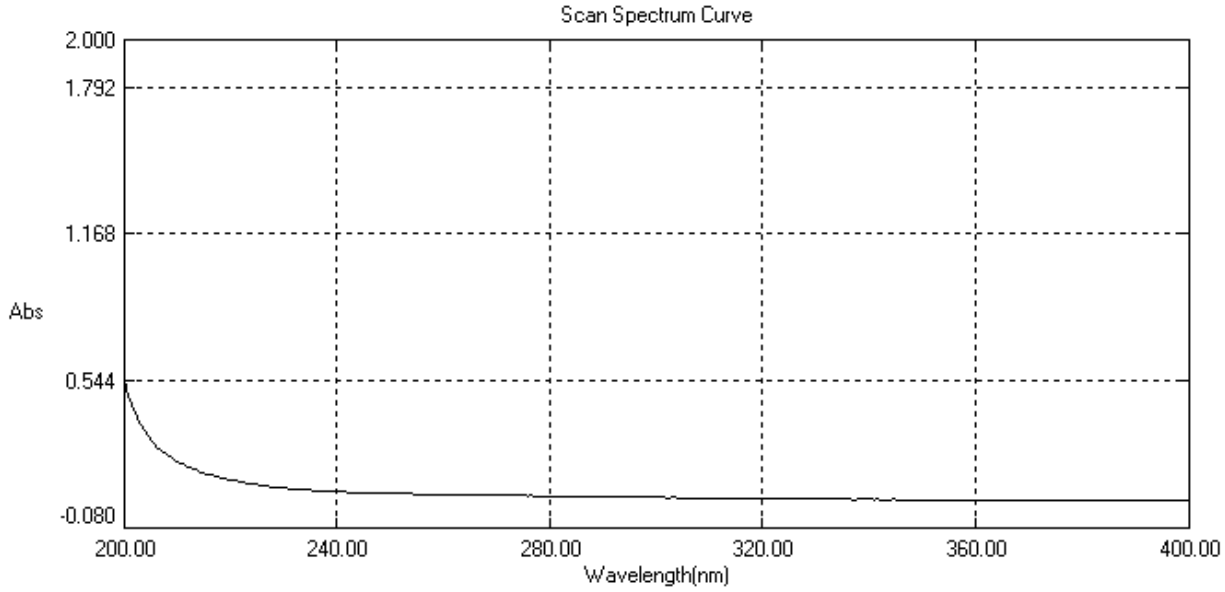
Appendix 6: FTIR spectrum of erythromycin A imino ether (KBr disk)



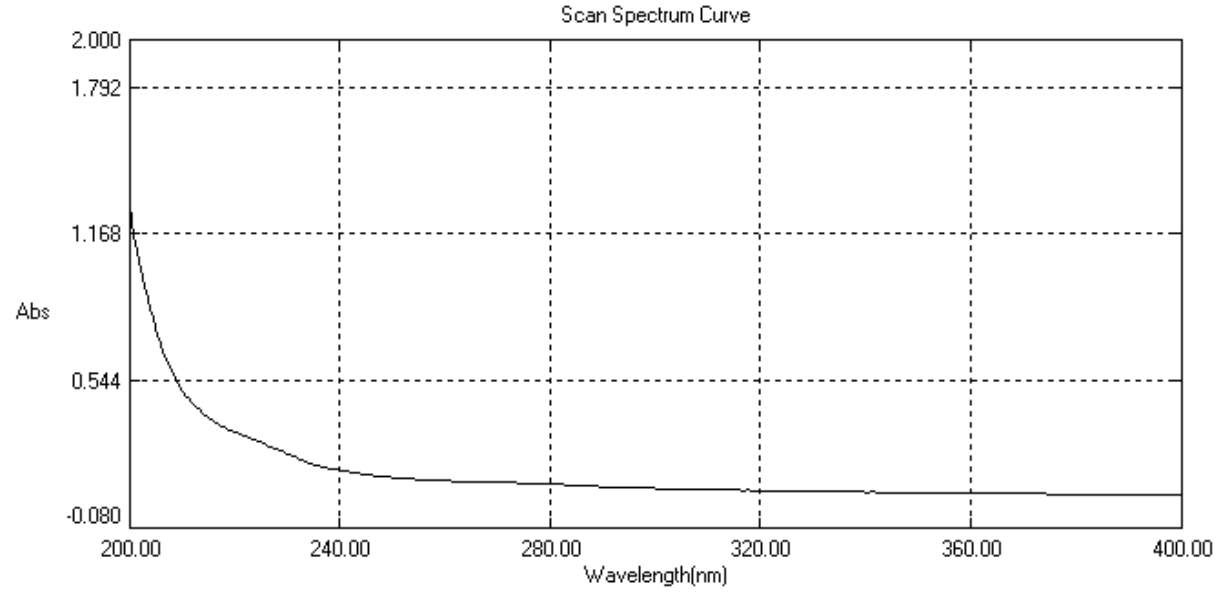
Appendix 7: UV spectrum of azithromycin standard



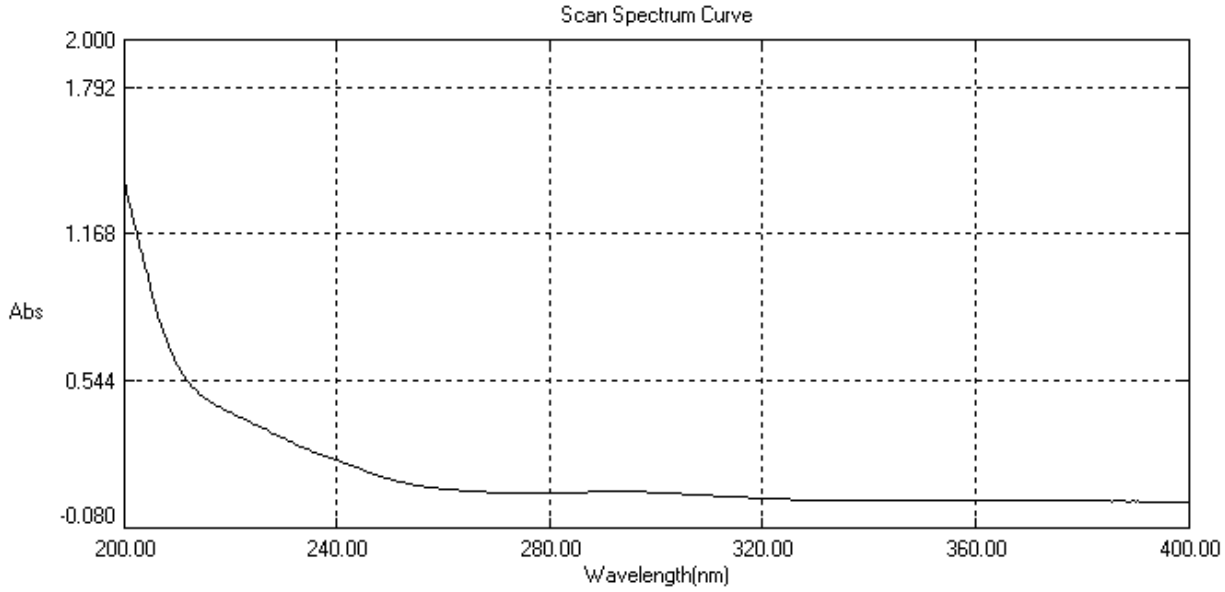
Appendix 8: UV spectrum of azathromycin CRS



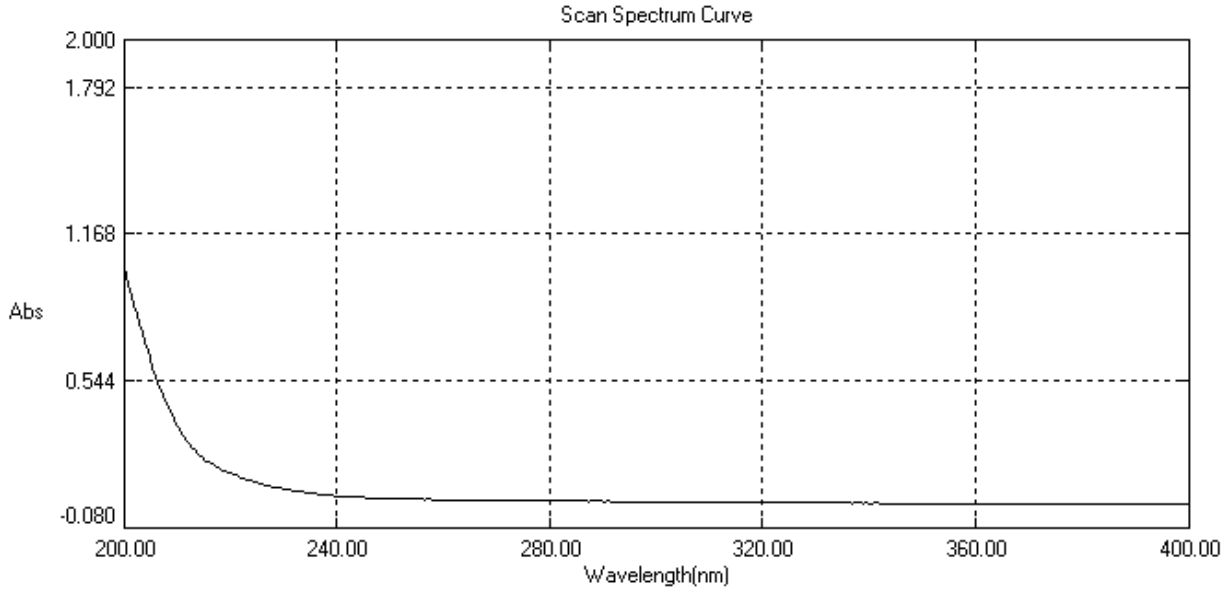
Appendix 9: UV spectrum of N-demethylazithromycin CRS



Appendix 10: UV spectrum of decladinosylazithromycin CRS



Appendix 11: UV spectrum of erythromycin A oxime CRS



Appendix 12: UV spectrum of erythromycin A imino ether CRS