Science Journal of Analytical Chemistry
2024; 12(1): 1-6
http://www.sciencepublishinggroup.com/j/sjac
doi: 10.11648/j.sjac.20241101.11
ISSN: 2376-8045 (Print); ISSN: 2376-8053 (Online)

Quality Control by HPLC of Azithromycin Specialties Marketed in Ivory Coast During COVID-19

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To cite this article:

Marcelle Adjoa Yao, Andre Sawa Kpaibe, Thodhekes Jean Simon Yao, Nina Able, Nicaise Bony et al. (2024). Quality Control by HPLC of Azithromycin Specialties Marketed in Ivory Coast During COVID-19. *Science Journal of Analytical Chemistry*, *12*(1), 1-6. https://doi.org/10.11648/j.sjac.20241101.11

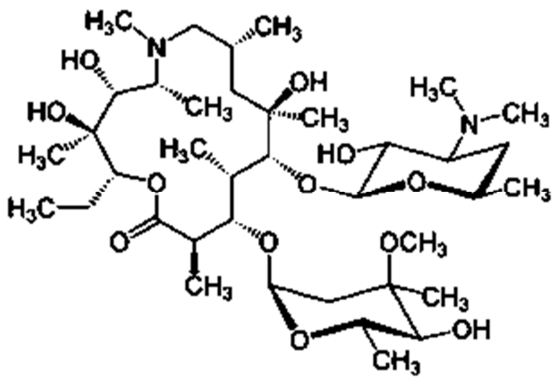
Received: November 20, 2023; **Accepted**: December 19, 2023; **Published**: January 8, 2024

Abstract: Azithromycin is an antibiotic of the macrolide subclass. Ivory Coast, in its strategy to combat the Covid-19 pandemic, has developed a treatment protocol including azithromycin. Faced with the high consumption of this molecule in the Covid-19 period and with the aim of fighting against substandard drugs, this work was undertaken in order to validate an HPLC method for the dosage of AZT and the search for its degradation products. The validated method was carried out on an octadecylsylanized column (250 mm × 4.6 mm, 5 µm) using a mobile phase which consisted of a methanol/phosphate buffer mixture at pH 7.5 (80/20 v/v). The flow rate was set at 1.5 ml/min for a column temperature of 50°C and the UV detection wavelength at 210 nm. Forced degradation studies were carried out under the conditions: acidic, alkaline, oxidizing, UV light and thermal, in order to determine the degradation profiles. The validation parameters which are the linearity domain, the limits of detection and quantification; repeatability and accuracy were satisfactory. All samples showed consistent azithromycin contents. However, related substances and degradation products have been found in certain specialties. The validation of the HPLC method gave reliable results which allowed the determination of AZT and the search for degradation products and impurities.

Keywords: COVID-19, Azithromycin, Validation, HPLC, Degradation Products

1. Introduction

On March 12, 2020, the WHO declared the COVID-19 outbreak a pandemic [1]. From the start of this pandemic until its end, there were a total of 666,599 confirmed cases and 6,864,100 deaths, with 170 countries affected in 5 continents [2]. Ivory Coast, like other countries in the world affected by this pandemic, has developed a treatment protocol following WHO recommendations. This protocol was based on prevention measures by wearing masks, physical distancing, hand washing, and the treatment of positive patients [3]. In a context of countries with limited resources, associated with supply difficulties, the government authorized the use of macrolides, in particular azithromycin (AZT), certainly based on the supposed anti-inflammatory and antiviral properties of this molecule which also part of immunomodulatory drugs of action in various respiratory diseases [4].



Scheme 1. *Chemical structure of azithromycin.*

Indeed AZT (scheme 1), antibiotic of the macrolide subclass, derived from erythromycin, from which it differs by the addition of a nitrogen atom [5], is one of the most used molecules during the COVID- 19 pandemic [6]. However, this use is the subject of controversy. Although some studies have shown its efficiency and benefits [7, 8]; others, on the contrary, advise against its use alone or in combination [9-12].

Furthermore, AZT, its degradation products and its impurities have been the subject of numerous analyzes in different biological matrices [13-15]; in the environment [16, 17], as well as its photocatalytic degradation by titanium dioxide [18].

The analysis methods implemented use increasingly complex techniques with sophisticated equipment. HPLC-MS; FITR [19-21].

Faced with the high consumption of AZT in the Covid-19 period and with the aim of combating substandard drugs, this work was undertaken in order to validate an HPLC method for the dosage of AZT and the search for its degradation products.

2. Materials and Methods

2.1. Reagents and Reference Chemical Substance

The reagents used were of analytical grade. They are composed of: anhydrous monobasic potassium phosphate, sodium hydroxide, 3% hydrogen peroxide, 1M hydrochloric acid, 1M sodium hydroxide; acetonitrile and Azithromycin of EDQM origin from the European Pharmacopoeia lot 4.0 with a purity of 94%.

The analyzes were carried out on 7 specialties of azithromycin tablets, 4 of which were dosed at 250 mg and 3 at 500 mg. In total there are 4 bottles of 500 bulk tablets dosed at 250 mg and 10 boxes of 3 tablets for each batch of tablets dosed 500 mg. These samples came from the official drug distribution circuit.

2.2. Equipment and Operating Conditions

The equipment consisted of an HPLC chain (Waters alliance e 2695/2998 PDA); a VWR USC-TH ultrasonic bath; an analytical balance; a pH meter (Mettler Toledo), filter paper and syringe filter with diameters of 0.45 µm; an oven; and a UV lamp.

Liquid chromatograph analyzes were carried out using the method taken from the literature [22]; on a stationary phase consisting of an octadecylsylanized column (250 mm x 4.6 mm.5 µm). The mobile phase consisted of a mixture of methanol and phosphate buffer at pH 7.5 (8/2 v/v), for a flow rate of 1.5 ml/min, an injection volume 20 µl, the temperature of the column was set at 50°C and detection in UV 210 nm.

The analyzes were carried out at the National Public Health Laboratory (LNSP).

2.3. Preparation of Solutions

2.3.1. Preparation of the Mobile Phase

The mobile phase consisted of a mixture of methanol and phosphate buffer at pH 7.5 in the proportions (80/20). This buffer was prepared by dissolving 4.55 g of potassium hydrogen phosphate in a 1000 ml flask containing purified water. The pH was adjusted with 10% sodium hydroxide solution.

2.3.2. Preparation of the Reference Solution

An amount of 100.2 mg of azithromycin powder was weighed and dissolved in a 50 ml vial containing mobile phase. The mixture was heated in an ultrasonic bath for 20 min. The concentration of the solution was 2 mg/ml from this mother solution (SM), a range of daughter solutions (SF) with concentrations of 0.6 mg/ml was prepared; 0.8 mg/ml; 1mg/ml; 1.2 mg/ml and 1.4 mg/ml. The SF concentrated at 0.6 mg/ml was prepared by taking 6 ml of the MS then diluted in a 20 ml flask with the mobile phase up to the mark. Those of SF dosed at 0.8 mg/ml 1 mg/ ml, 1.2 mg/ ml; and 1.4 mg/ml were prepared by diluting with the mobile phase in respective 10 ml vials of 4 ml, 5 ml, 6 ml, and 7 ml of SM.

2.3.3. Preparation of Solutions to Analyze

(i). Preparation of Non-Degraded Solutions

Weigh and pulverize 20 azithromycin tablets. Take a quantity of powder corresponding to 50 mg of azithromycin and dissolve it in approximately 40 ml of mobile phase for 20 min using ultrasound then make up to the mark with the same solvent and filter with a 0.45 µm syringe filter.

(ii). Preparation of Degraded Solutions

The raw material (MP) of azithromycin was subjected to 5 forced degradation conditions namely: acidic, basic, oxidative, ultraviolet (UV) rays and heat.

1) Preparation of MP degraded with strong acid: in a 50 ml volumetric flask, 50 mg of AZT powder, 1 ml of 0.5M hydrochloric acid were introduced, after 2 hours the mixture was neutralized with 1L of 0.5M sodium hydroxide, then the mixture was made up to the mark with the mobile phase [23].
2) Preparation of degraded MP with a base: in a 50 ml volumetric flask, 50 mg of AZT powder were introduced, 1 ml of 0.1M sodium hydroxide was added, after 2 minutes the mixture was neutralized with 1 ml of 0.1M hydrochloric acid, then the mixture was made up to the mark with the mobile phase [23].
3) 3% hydrogen peroxide solution (H_2O_2), after 30 minutes, the mixture was completed to the gauge mark with the mobile phase [23].
4) Preparation of heat-degraded MP: 50 mg of AZT powder was placed in the oven at 85°C for 3 hours, then dissolved with the mobile phase [23].
5) Preparation of MP degraded with UV light at 254 nm: 50 mg of AZT powder was exposed to UV rays for 1 hour, then dissolved with the mobile phase [24].

2.4. Validation Criteria

Validation parameters included linearity, repeatability, accuracy, Limit of Detection (LOD) and Limit of Quantification (LOQ). All tests were carried out with

solutions prepared from the reference substance azithromycin.

The linearity study focused on 5 concentration levels (0.6 mg/l; 0.8 mg/l: 1 mg/l: 1.2 mg/l; 1.4 mg/l). Repeatability covered 2 concentration levels (80% and 120%) corresponding to 0.8 mg/ml and 1.2 mg/ml respectively. Concerning the accuracy of increasing amounts of azithromycin i.e. (80.02; 100.36 and 120.06) mg were added to an initial amount of 15 mg and the recovery percentages were determined.

The limits of detection and quantification were obtained by calculation using the calibration line according to the formulas below.

$$LOD = 3.3\ \sigma / S$$

$$LOQ = 10\ \sigma / S$$

where σ: is the standard deviation and S: is the slope of the linearity line

3. Results

3.1. Validation of the Analysis Method (Table 1)

The analysis of AZT by High Performance Liquid Chromatography (HPLC) under the defined conditions allowed the detection of AZT in less than 5 minutes.

A domain of linearity was highlighted in the AZT concentration range from 0.6 mg/l to 1.4 mg/l with a regression line of equation Y= (9963.5± 3104.6) X +(-50840±29452) and a coefficient of determination $R^2 >$ 0.99 (table 1).

Repeatability (n=6) on concentrations 0.8 mg/l (0.44%) and 1.2 mg/l (1.27%) gave CVs of < 5% (reference value) [25].

The limits of detection and quantification were respectively 56.18 µg/ml and 76.78 µg/ml. (Table 1)

The accuracy determined by the recovery percentage was 99.91%. (Table 2)

Table 1. *Result of validation of the analysis method.*

Settings	Result
Linearity	Y= (9963.5 ± 3104.6) X + (-50840 ± 29452) R2 = 0.996
LOD mg/ml	0.0986
LOQ mg/ml	0.2989
Repeatability 80%	
RSD%	0.44
Repeatability 120%	
RSD%	1.27

Table 2. *Accuracy results of chromatographic method.*

Initial AZT Quantity (mg)	Quantity AZT added (mg)	Total AZT quantity measured (mg)	Recovery percentage (%)
15.00	80.02	95.11	100.13
15.00	100.36	115.85	99.65
15.00	120.08	135.00	99.95
Average recovery percentage			99.91

3.2. Applications

The method thus validated was used for the dosage of 7 specialties of AZT tablets. AZT was identified in all samples analyzed. The average AZT levels obtained were all within the defined compliance interval of 450 mg to 550 mg for tablets dosed at 500 mg, and 225 to 275 mg for those dosed at 250 mg. The contents obtained were recorded in Table 3.

Forced degradation of AZT raw material under acidic conditions; basic; oxidative; thermal, and ultraviolet, allowed to determine the different degradation profiles of AZT. These degradation profiles were presented in Figure 1. The analysis of the chromatograms of the samples made it possible to identify 2 impurities, including N- Dimethylazithromycin which was found in 6 samples out of the 7 analyzed and the impurity P which was found in only one sample (Table 3).

No interference between azithromycin and its degradation products has been observed. Forced degradation conditions in an acidic environment led to rapid decomposition of AZT; similar results were obtained with Fiese et al., who showed that azithromycin is rapidly decomposed in acidic solution by intra-molecular dehydration to the form of erythromycin-6,6-hemiceta and subsequently anhy erythromycin-6,6-hemiketalan and thenanhydroerythromycin [26].

The analysis of the chromatograms of the samples made it possible to identify 2 impurities, including N-Dimethylazithromycin which was found in 6 samples out of the 7 analyzed and the impurity P which was found in only one sample [27].

On the other hand, an unknown peak was determined in a sample with a retention time of 6 minutes; this peak could come from an excipient in the formulation.

Table 3. *Results of the analysis of azithromycin specialties.*

Sample	Content in AZT (mg/tablet)	% AZT	Impurity AZT	AZT degradation product
S1	518.51	103.7	N –Dimethyl azithromycin	Presence of unknown peak
S2	494.44	98.89	N -Dimethylazithromycin	Absence
S3	467.49	93.5	Absence	Absence
S4	254.5	101.8	N -Dimethylazithromycin	Absence
S5	255.37	102.15	N- Dimethylazithromycin + Impurity P	Absence
S6	252.79	101.11	N -Dimethylazithromycin	Absence
S7	255.02	102.01	N -Dimethylazithromycin	Absence

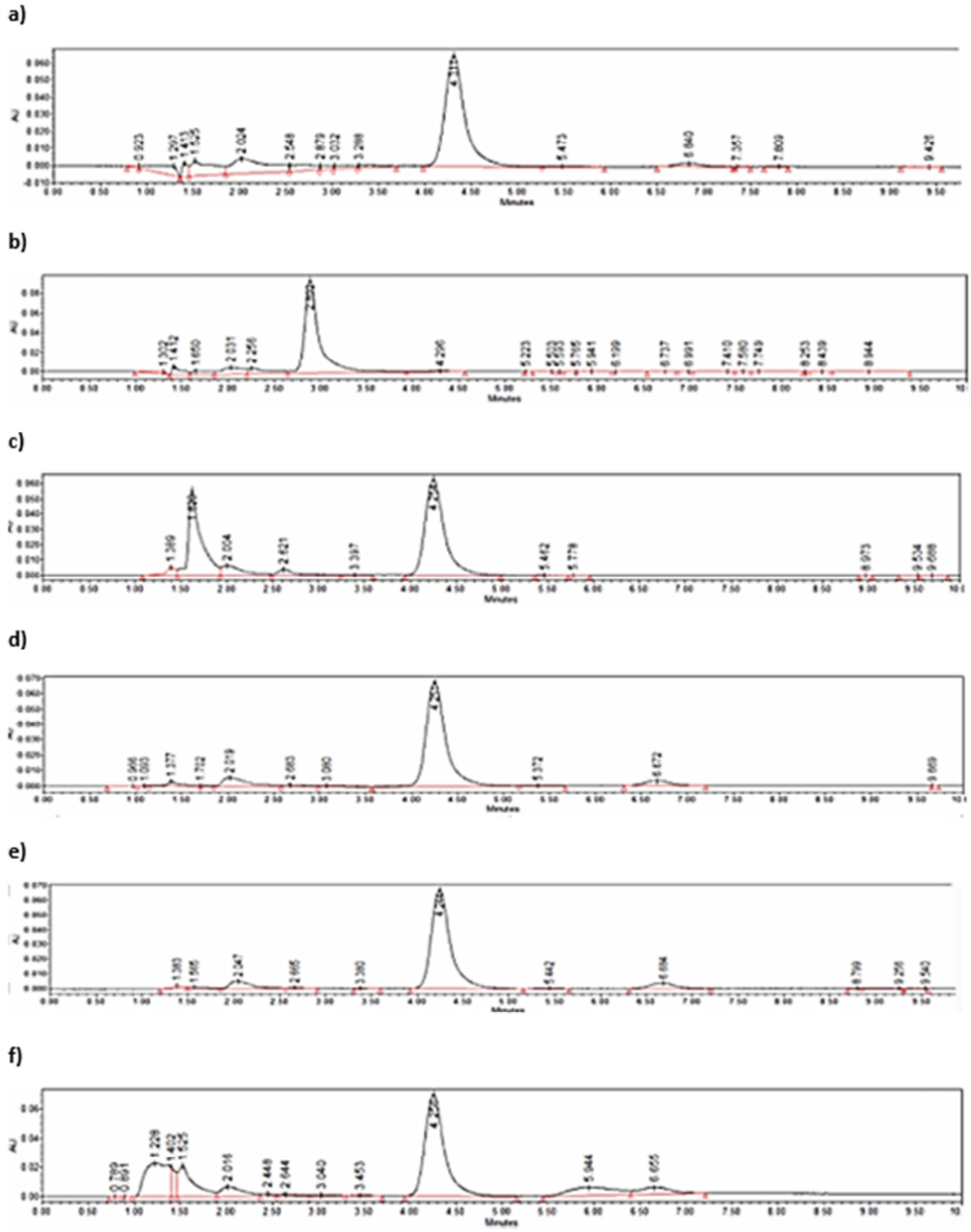


Figure 1. *Degradation profiles of AZT solutions (1mg/mL) and chromatogram of non-degraded sample S1.*

The Figure 1 presents the degradation profiles of MP with a) degradation with 0.1 M of NaOH for 2 min, b) degradation with 0.5 M HCl for 2 h; c) degradation with 3% of hydrogen peroxide for 30 min; d) degradation by UV rays for 1 hour, at 254 nm and e) degradation by heat at 85°C for 3 hours; f) chromatogram of sample S1 non- degradation.

4. Discussion

The analysis of AZT by High Performance Liquid Chromatography (HPLC) under the defined conditions allowed the detection of AZT in 4.5 minutes, unlike the work of Mostafa who obtained a longer retention time (5, 8 minutes), with a Methanol/phosphate buffer (9/1) v/v mobile phase.

This reduction in analysis time will contribute to reducing analysis costs in the context of routine control [28].

In accordance with ICH requirements, the method validation criteria of linearity, repeatability, accuracy, LOD, and LOQ were satisfactory, making this method suitable for the determination of AZT tablets [25].

AZT was identified in all samples analyzed. The average AZT levels obtained were all within the compliance interval defined by the American Pharmacopoeia, i.e. 450 mg to 550 mg for tablets dosed at 500 mg, and 225 to 275 mg for those dosed at 250 mg [27]. No interference between AZT and its degradation products has been observed. Forced degradation conditions in an acidic environment led to rapid decomposition of AZT; similar results were obtained with Fiese and al., who showed that azithromycin is rapidly decomposed in acidic solution by intra-molecular dehydration to the form of erythromycin-6,6-hemiceta and subsequently anhy erythromycin-6,6-hemiketalan and thenanhydroerythromycin [26].

The analysis of the chromatograms of the samples made it possible to identify 2 impurities, including N-Dimethylazithromycin which was found in 6 samples out of the 7 analyzed and the impurity P which was found in only one sample (S5) [27].

On the other hand, an unknown peak was determined in a sample with a retention time of 6 minutes; this peak could come either from an excipient of the formulation or be a degradation product of AZT obtained under conditions other than those carried out in our study.

These results showed the importance of extending the quality control of AZT, to research and even quantification of its degradation products and impurities. In fact, its impurities at high levels could affect the quality of drugs and subsequently lead to drug resistance phenomena.

5. Conclusions

This work made it possible to validate a chromatographic method for dosing oral forms of azithromycin marketed during Covid-19 in Côte d'Ivoire. The validation of the analysis method gave satisfactory results which allowed the analysis of 7 azithromycin specialties. At the end of our analysis, we noted the conformity of the azithromycin content of all the samples. However, some impurities were found in some samples. This validated method could be used in routine quality control of azithromycin and its degradation products to detect counterfeit or substandard drugs.

Acknowledgments

We would like to thank Mr. Attié Yves Landry, pharmacist and Mr. Suy Bi Benjamin, technician at the National Public Health Laboratory for their technical support for this work.

Conflicts of Interest

The authors declare no conflict of interest.

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