

Development of a Thin Layer Chromatography Method for Simultaneous Detection of Chloroquine, Azithromycin and Paracetamol in Counterfeit Tablets Used Against Covid-19

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Abstract: Corona Virus Disease 2019 (Covid-19) is an infectious disease manifested by fever, rate and difficulty in breathing that can lead to death. This pandemic has just paralyzed the world, causing more deaths amongst vulnerable people such as the elderly and people with immune problems. Several therapeutic agents are being tested for the treatment of Covid-19. The combination of Chloroquine, Azithromycin and Paracetamol is identified as a promising therapeutic candidate for Covid-19. To ensure the success of this therapy, medicines of high quality play a key role. Unfortunately, we are not totally spared from trafficking of poor quality pharmaceutical products. As a contribution to try solving this challenge, a convenient, rapid and simple Thin Layer Chromatography method (TLC), which permits the simultaneous determination of Chloroquine, Azithromycin and paracetamol has been developed. The Chromatographic separation was achieved on 60 F silica gel plate using a mixture of methanol-25% ammonia (100:1.5, v/v). Iodine vapor and ultraviolet light at 254 nm were used for the visualization of the spots. The developed method was successfully applied to determine Chloroquine in 23 samples supposed to contain Chloroquine marketed in DRC. The results showed that 50% of the samples analyzed were non-compliant and should probably contain paracetamol instead of Chloroquine. It suggested that the optimized method could be used for routine quality assessment of Chloroquine, Azithromycin or Paracetamol in pharmaceutical preparations. Since counterfeiting of medicines continues to be a real problem in developing countries as demonstrated by this result, the development of simple and convenient analytical method that allowed the rapid analysis of medicines used in Covid-19 therapy is more than urgent.

Keywords: Thin Layer Chromatography, Simultaneous Detection, Counterfeit Tablets, Covid-19

1. Introduction

Coronavirus disease 2019 or Covid-19 (acronym for coronavirus disease 2019) is an emerging infectious disease like viral zoonosis caused by a strain of coronavirus called SARS-CoV-2 [1-5]. The most common symptoms are fever, cough, and breathing difficulty and more rarely, acute respiratory distress syndrome that may lead to death,

especially amongst vulnerable people such as the elderly, hypertensive and diabetics patients. Another deadly complication is an exacerbated immune system response [1-3].

The disease appears in November 2019 in Wuhan, central China with unusual cases of pneumonia justifying severe containment measures in January 2020 [6-11]. In March 2020, the epidemic was reclassified as a pandemic by the World Health Organization (WHO) [4]. The Covid-19 pandemic

spread rapidly to many other countries, which in turn took similar action in March, causing border closures, quarantining citizens and cancelling large gatherings such as concerts, schools and sporting events, as in the Democratic Republic of Congo (DRC).

Recommended measures to prevent Covid-19 include frequent hand washing using detergent or hydroalcoholic solution, face coverings, quarantine and social distancing. One of the therapeutic options is based on the rapid and correct management of clinical cases by administering Chloroquine or Hydroxychloroquine combined with Azithromycin and Paracetamol in case of fever. For the researcher Didier Raoult, director of the Mediterranean Infection Institute in Marseille (France) and renowned specialist in infectious diseases, the possible effectiveness of Chloroquine is "excellent news" in the treatment of covid-19 [1-4].

Chloroquine is no longer used to prevent nor treat malaria in DRC because of the selection of *Plasmodium falciparum* resistant isolates. As a consequence, Chloroquine is no longer available in public pharmacies. In response to this pandemic, the need for Chloroquine is increasing and counterfeiters are

taking the opportunity of launching drugs of doubtful quality on the market, especially in developing countries where the quality system remains precarious. Such practice may totally compromise the success of this therapeutic option vis-a-vis Covid-19. The WHO launched an alert on April 9, 2020 on the falsification of Chloroquine in certain African countries notably Cameroon, Niger and the DRC [12].

In this context, a simple analytical technique capable of simultaneously detecting these molecules (Chloroquine or Hydroxychloroquine, Azithromycin and Paracetamol) will be desirable in quality control laboratories that do not have advanced techniques such as High Performance Liquid Chromatography. Keeping this in mind, TLC which is low cost and less time consuming separative technique was developed in this study.

The aim of this study was mainly to develop a generic TLC method capable of simultaneously tracing these three active molecules with the goal of detecting them, highlighting falsifications or counterfeits and secondly demonstrate its applicability on some real samples collected in DRC

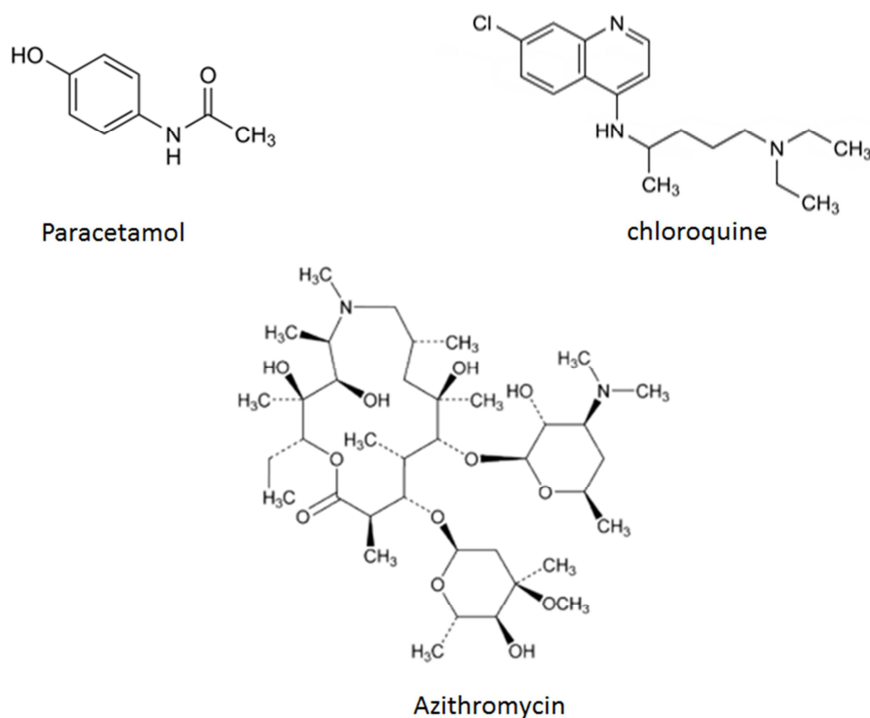


Figure 1. Chemical structures of studied molecules.

2. Experimental

2.1. Material

The analysis was carried out using ready-to-use TLC plates, silica gel 60F (20 × 20 cm) from the company Merck (Darmstadt, Germany). These plates were placed in glass vats. The electronic scale was of the GRAM FV-220C brand (IPESAGE S. A. S, France). A small amount of samples and standard were loaded to a starting point just above the bottom of TLC plate by using a 10 µL micropipettes. The

development of the TLC plate was carried out by using tank with filter paper liners and by leaving the tank to equilibrate before running the plates.

2.2. Chemical Reagents

Methanol, chloroform, 98% acetic acid and 25% ammonia for analysis were supplied by the Merck laboratory (Darmstadt, Germany). Chloroquine diphosphate (99.2%) was supplied by Sigma – Aldrich (Antwerp, Belgium), Paracetamol (99.5%) was supplied by Fagron N. V. (Waregem, Belgium). Azithromycin, corn starch, carboxymethylcellulose,

magnesium stearate, talc, lactose and gelatin were supplied by the Laboratory for Quality Control of Medicines and Foodstuffs (Kinshasa, DRC).

2.3. Methods

2.3.1. Preparation of Solutions

- 1) Chloroquine phosphate solution 10 mg mL⁻¹
100 mg of Chloroquine phosphate were dissolved in acetic acid and diluted to 10.0 mL with the same solvent.
- 2) Azithromycin solution 10 mg mL⁻¹
100 mg of Azithromycin were dissolved in methanol and diluted to 10.0 mL with the same solvent.
- 3) Paracetamol solution 10 mg mL⁻¹
100 mg of Paracetamol were dissolved in methanol and diluted to 10.0 mL with the same solvent.
- 4) Mixing solution
In a 10.0 mL volumetric flask 2.0 mL of Chloroquine phosphate solution, 2.0 mL of Azithromycin solution and 2.0 mL of Paracetamol solution were mixed.
- 5) Matrix blank solution
100 mg of corn starch, 100 mg of carboxymethylcellulose, 100 mg of magnesium stearate, 100 mg of talc, 100 mg lactose and 100 mg of gelatin were successively dissolved in methanol and diluted to 100.0 mL with the same solvent. The mixture was filtered and the filtrate used for the study.

2.3.2. Preparation of Mobile Phases

Phase 1: 1.5mL of 25% ammoniac and 100 mL of methanol were mixed in a 250 mL beaker

Phase 2: 1.5 mL of 25% ammoniac, 20 mL of methanol and 80mL of chloroform were mixed in a 250 mL beaker.

Phase 3: 80 mL of chloroform and 20 mL of methanol were mixed in a 250 mL beaker.

2.3.3. Procedure

After dissolution of the active ingredients, 10 µL of each solution were deposited using a micropipette on the plate (previously activated) 2 cm from the bottom edge on the baseline. Each deposit was dried. The plate was then placed in the migration chamber containing the mobile phase. When the solvent front reached 1 cm from the upper edge of the plate, the chromatograms were removed, dried and viewed in the UV lamp at 254 nm and then using iodine steam.

3. Results and Discussion

3.1. Method Developed

To fully appreciate the separation of the three active ingredients, we used a random experiment plan which takes into account two factors, the concentration of active ingredient and the composition of the mobile phase. Table 1 gives the experimental domain. The experimental matrix and the results obtained during the experiments are depicted in Table 2. The minimum front gear is the difference between the closest front gears.

Table 1. Experimental domain.

Factors	Levels		
Concentration (mg/mL)	0.1	1	10
Mobile phase	Phase 1	Phase 2	Phase 3

Table 2. Experimental matrix.

Trial	Concentration (mg. mL ⁻¹)	mobile Phase	Retention factor			minimal retention factor
			Chloroquine	Azithromycin	Paracetamol	
1	0.1	Phase 1	-	-	-	-
2	0.1	Phase 3	-	-	-	-
3	10.0	Phase 3	-	-	-	-
4	10.0	Phase 2	0.35	-	0.76	0.41
5	1.0	Phase 3	-	-	-	-
6	1.0	Phase 2	-	-	-	-
7	0.1	Phase 2	-	-	-	-
8	1.0	Phase 1	0.35	-	-	-
9	10.0	Phase 1	0.35	0.51	0.75	0.16

Legend: spot not detect.

Good separation of the three active pharmaceutical ingredients with the minimum retention factor value greater than zero was found with test 9 using phase 1, methanol-25% ammoniac (100:1.5, v/v) at a concentration of 10 mg mL⁻¹ (Figure 1). The used mobile phase was selected because of its good polarity and its capacity to easily separate the medicines under analysis. Paracetamol was the most soluble (Log P=0.5), moved higher up the plate than Azithromycin (Log P=3.3) and Chloroquine (Log P=4.6) [13]. Given three compounds which differ in solubility, the more soluble compound (Paracetamol) has a lower interaction with the TLC plate and is therefore more capable to move higher up the plate, resulting in a higher Rf value.

The analytes concentration played an important role, this is why the spots were clearly visible at high concentration such as 10 mg mL⁻¹ which was not the case for 0.1 mg mL⁻¹.

The minimum retention factor (Rf) value obtained was 0.16 or greater than 0.00 which means that the spots were well separated. After having saturated the chromatographic tank, 25 minutes were enough to obtain separation.

The developed method has been validated by demonstrating the selectivity as required by the French Societies in Pharmaceutical Sciences and Technologies [14-16]. There was no interference with the white matrix (without active ingredients) in the frontal relationships of the active molecules.

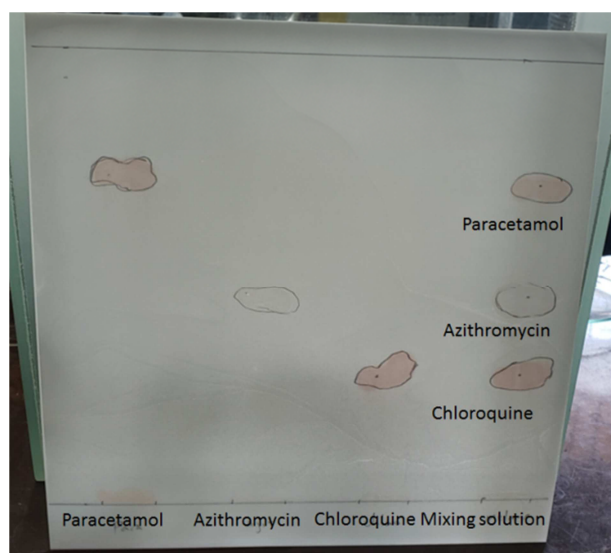


Figure 2. Chromatogram of separation of the three active ingredients by TLC.

3.2. Method Application

We applied the developed method by analyzing 23 samples marketed in Kinshasa (DRC) as shown in Table 3.

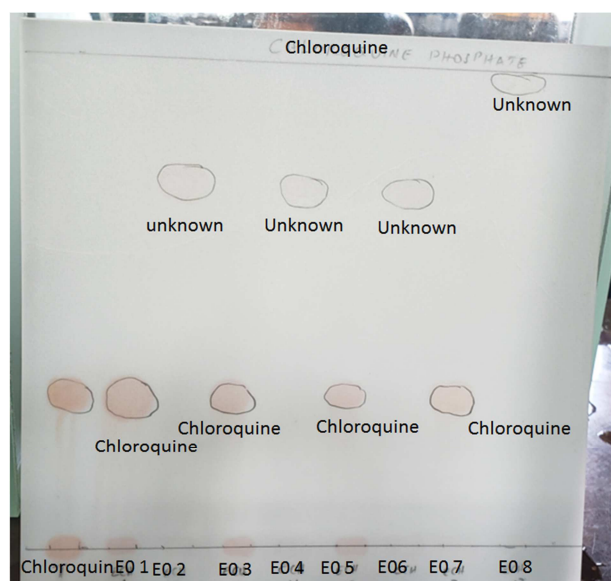


Figure 3. Analysis chromatogram of 8 samples based on chloroquine by TLC.

Table 3. Table depicting the samples characteristics.

Codes	Actif ingredient	Tablet dosage	Batch number	Country of origin
E01	Chloroquine	250 mg	00214L	India
E02	Chloroquine	250 mg	028060	Nigeria
E03	Chloroquine	250 mg	065622	Belgium
E04	Chloroquine	250 mg	EBT 2543	Cameroon
E05	Chloroquine	250 mg	EBT 172522	Austral
E06	Chloroquine	250 mg	660	China
E07	Chloroquine	100 mg	11220A	India
E08	Chloroquine	250 mg	HV1117	No indiqued
E09	Paracétamol	500 mg	121103	DRC
E10	Paracétamol	500 mg	N9285	Belgium
E11	Paracétamol	500 mg	DAL 4Q09	Belgium
E12	Paracétamol	500 mg	N7689	Belgium

Codes	Actif ingredient	Tablet dosage	Batch number	Country of origin
E13	Paracétamol	500 mg	5289	French
E14	Paracétamol	500 mg	Ji 20100007	China
E15	Paracétamol	500 mg	TG 00297	DRC
E16	Paracétamol	500 mg	TG 00276	DRC
E17	Azithromycin	500 mg	1711219	India
E18	Azithromycin	500 mg	831700	India
E19	Azithromycin	500 mg	ATAZM 8008	India
E20	Azithromycin	500 mg	116578L	India
E21	Azithromycin	500 mg	12202BS	India
E22	Azithromycin	500 mg	1A11002	India
E23	Azithromycin	500 mg	20223A1	India

The overall analysis (identification of active molecules) carried out on the real samples showed that 4/23 (or 17.4%) of the products were counterfeit, especially 4/8 or 50% of Chloroquine failed the identification test (Figure 2).

While 100% of Paracetamol and Azithromycin samples complied to the identification test. Counterfeiting medicines is very dangerous in the treatment of Covid-19 with risk of therapeutic failure and death for patients who are in critical situations.

The samples E02, E04 and E06 showed spots with Rf values closed to Rf value for Paracetamol (i.e. 0.75) while sample E08 showed a spot with an Rf value of 0.98. HPLC-UV was used to identify the actives pharmaceutical ingredients in those samples (E02, E04 and E06) by comparing their UV spectra and retention times to those of Paracetamol reference substance. The UV spectra and retention times of those samples matched with the UV spectrum and retention time of Paracetamol reference solution (data not available in this study). This is to say that Paracetamol could be the probable product used in these samples (E02, E04 and E06) instead of Chloroquine. Extensive studies should confirm this observation. The counterfeiters have bet on the antipyretic product used against Covid-19 which costs less to earn money and treat only fever, so one could avoid using dangerous products like Anti-inflammatory No Steroids.

4. Conclusion

The main goal of this study was to develop a simple, rapid and convenient method for the simultaneous detection of Chloroquine, Azithromycin and Paracetamol used in the treatment of Covid-19 to fight against the circulation of counterfeit medicines during this pandemic.

A simple method using the 60F silica gel plate, 20 x 20 cm in size with a mobile phase composed of methanol-25% ammonia (100:1.5, v/v) has been developed. The optimized method was applied to determine Chloroquine, Azithromycin and Paracetamol in real samples tablets. Using HPLC-UV, it was demonstrated that E02, E04 and E06 were probably containing Paracetamol instead of Chloroquine, which confirmed the WHO alert on counterfeit products found in the WHO African regions.

Counterfeit medicine is a threat in the fight against covid-19. We hope that this simple and rapid detection method will be

helpful for a large number of laboratories in developing countries to ensure the quality of medicines currently used in the treatment of Covid-19.

In fine, the study results illustrated and highlighted the opportunity to use Thin Layer Chromatography which is low cost, less time consuming, less complicated separation technique and currently found in several laboratories, particularly in developing countries for the simultaneous analysis of Chloroquine, Azithromycin and Paracetamol.

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