Mateus Araújo Castro e Souza1,*, Naialy Fernandes Araújo Reis1, Larissa de Souza Batista1, Isabela da Costa César1, Christian Fernandes1 and Gerson Antônio Pianetti1

1Quality Control Laboratory for Medicines and Cosmetics, Department of Pharmaceutical Products, Faculty of Pharmacy, Federal University of Minas Gerais, Belo Horizonte, Brazil

Abstract: Introduction: Malaria, an infectious disease caused by protozoa of the genus Plasmodium, is highly prevalent in the Brazilian Amazon. Chloroquine is the first-choice drug for the treatment of malaria caused by *P. vivax* and *P. malariae*. The humid and hot climate characteristic of the Brazilian endemic region favors drug degradation and modification of its biopharmaceutical properties, which may result in subtherapeutic dosage, formation of degradation products that can be toxic to humans and appearance of parasitic resistance. Thus, it is necessary to monitor the quality of chloroquine tablets.

Materials and Methods: An analytical method was developed and validated to determine chloroquine content in tablets by ultraviolet spectrophotometry. The diluent consisted of 0.06 M monosodium phosphate buffer pH 6.8 and detection was performed at 343 nm.

Results and Conclusion: The method proved to be linear in the range of 7.2 to 19.2 µg.mL⁻¹, precise, accurate, selective, robust, and statistically equivalent to a liquid chromatographic method by the United States Pharmacopeia. The developed method was applied to determine chloroquine content in six batches of the drug. The evaluated batches were considered adequate for identification, assay, dissolution, disintegration and uniformity of dosage units, and were found to be inadequate in terms of friability.

Keywords: Malaria, chloroquine diphosphate, tablets, ultraviolet spectrophotometry, analytical method development, quality control.

1. INTRODUCTION

Malaria is the most common and important infectious disease in humans. It is estimated that around 3 billion people worldwide are at risk of contracting malaria, mainly in Asia, Africa and Central and South America. In Brazil, up to 20% of the population is at risk of infection [1].

The National Program for Malaria Control (PNCM), established by Brazilian government, aims to reduce fatality, severity and disease incidence, eliminate transmission in urban areas and maintain absence of disease in the regions where the transmission has already been interrupted. Through the PNCM, the Ministry of Health distributes drugs to control malaria in the Unified Health System, and chloroquine is the drug of first choice for the treatment of uncomplicated malaria [2].

Chloroquine diphosphate, chemically known as N4-(7-chloro-4-quinolinyl)-N1,N1-diethyl-1,4-pentanediamine diphosphate (Fig. 1), molecular mass of 515.86 g.mol⁻¹, is a 4-aminoquinoline commonly prescribed for the treatment of malaria [3, 4].

![Chemical structure of chloroquine diphosphate.](image)

Fig. (1). Chemical structure of chloroquine diphosphate.

The high temperature (average of 28 °C) and humidity (88% in the rainy season and 77% in the dry season), characteristic of the Brazilian malaria endemic region [5], favors the degradation and alteration of the biopharmaceutical properties of the drugs. This may result in sub-therapeutic dosage and formation of degradation products toxic to humans. Thus, the parasites are more likely to develop resistance to antimalarial and, therefore, the treatment goals may not be achieved [6, 7]. To ensure the efficacy and safety of
treatment, the continuous monitoring of antimalarial drugs quality is necessary.

Assay methods for chloroquine diphosphate in tablets have been already described in different pharmacopoeias. The Brazilian Pharmacopoeia [8] recommends two analytical methods for the determination of chloroquine diphosphate content, both involving previous extraction steps of the free base, one by ultraviolet spectrophotometry and other by nonaqueous titration. The International [9] and British [10] pharmacopoeias present nonaqueous titration methods, both also having a previous step of extracting the free base. The United States Pharmacopoeia [11] recommends a method by high performance liquid chromatography (HPLC). Besides pharmacopeial methods, other chromatographic approaches for chloroquine determination in pharmaceuticals have been described in the literature [12-14]. Although HPLC is a highly selective technique, it requires expensive instrumentation and trained technicians, which may be a limitation in areas where there is high malaria transmission, usually characterized by insufficient economic resources. Furthermore, HPLC is more time-consuming when compared to ultraviolet spectrophotometry [15].

In this context, this study aimed to develop and validate a rapid, simple, inexpensive and selective method to quantify chloroquine diphosphate in tablets by ultraviolet spectrophotometry and apply this method in real samples distributed by the Brazilian Unified Health System in the northern region of Brazil.

2. MATERIALS AND METHODS

2.1. Chemical, Reagents, and Materials

Chloroquine diphosphate reference standard (99.70% purity) was obtained from the United States Pharmacopeia (Rockville, MD, United States). Six batches of tablets containing 150 mg of chloroquine (equivalent to 241.91 mg of chloroquine diphosphate), from one supplier, were collected in northern Brazil, in the states of Roraima, Acre, Amapá, Rondônia, Pará and Amazonas. Corn starch, mannitol, talc and magnesium stearate were obtained from Valdequímica (São Paulo, SP, Brazil). Ultrapure water was from a Direct-Q 3 Millipore system (Bedford, MA, USA). Sodium hydroxide and monosodium phosphate (analytical grade) were purchased from Sigma-Aldrich (St Louis, MO, USA). Quantitative filter paper was purchased from J. Prolab (São José dos Pinhais, PR, Brazil).

2.2. Instrumentation

The ultraviolet spectrophotometric analyses were carried out on a Varian Cary 50 dual beam spectrophotometer (Palo Alto, CA, United States). The software Cary WinUV was employed for measurements, which were obtained with medium scan speed of 600 nm.min⁻¹, spectral bandwidth of 1.5 nm and a 10 mm optical path quartz cuvette. All spectra were recorded from 200 to 400 nm. UV detection was performed at 343 nm, with three scans per sample, using 0.06 M monosodium phosphate buffer pH 6.8 as blank.

The chromatographic analyses were carried out on a Waters Alliance (Milford, MA, USA), composed of an e2695 separation module and a 2998 UV/DAD detector. The chromatographic separation was performed on a Thermo Scientific Hypersil Gold C18 (100 x 4.6 mm i.d.; 5 µm particle size) column, maintained at 30 °C. The injection volume was 10 µL. The mobile phase was a mixture of 0.05 M monopotassium phosphate buffer pH 2.50 and methanol (78:22, v/v), at a flow rate of 1.2 mL.min⁻¹.

2.3. Preparation of Solutions

2.3.1. Diluent

Approximately 6.9 g of monosodium phosphate and 0.9 g of sodium hydroxide were weighed and transferred to a 1000 mL volumetric flask, followed by the addition of 800 mL of water. The pH was adjusted to 6.8 using 1.0 M sodium hydroxide solution. The flask was filled to the mark with water.

2.3.2. Standard Solutions

Approximately 12 mg of chloroquine diphosphate were accurately weighed and transferred to a 100 mL volumetric flask, followed by the addition of 80 mL of diluent. The flask was sonicated for 10 minutes and filled to the mark with diluent, obtaining a solution with concentration of 120.0 µg.mL⁻¹. Working solutions were prepared by proper dilution of the stock standard solution.

2.3.3. Tablet Sample Solutions

The average weight of 20 tablets containing 241.91 mg of chloroquine diphosphate was determined. The tablets were crushed, and the powder was further homogenized. An amount of powder equivalent to 60.48 mg of chloroquine diphosphate was transferred to a 100 mL volumetric flask, followed by the addition of 80 mL of diluent. The flask was sonicated for 20 minutes and was filled to the mark with the same solvent. The solution was filtered using a quantitative filter paper. An aliquot of 2 mL of the solution was transferred to a 100 mL volumetric flask. The flask was filled to the mark with diluent, obtaining a solution with theoretical concentration of 12.0 µg.mL⁻¹.

2.3.4. Placebo Solution

Approximately 45 mg of the mixture of excipients (60% corn starch, 33% mannitol, 2% talc and 5% magnesium stearate), prepared according to the qualitative composition provided by the supplier and the amount of excipients usually employed in tablets [16], were accurately weighed and transferred to a 100 mL volumetric flask, followed by the addition of 80 mL of diluent. The flask was sonicated for 20 minutes and filled to the mark with diluent. An aliquot of 1 mL of the solution was transferred to a 50 mL volumetric flask. The flask was filled to the mark with diluent.

2.4. Method Validation

The developed spectrophotometric method was validated according to the Brazilian Guideline RE N° 899/2003 [17] and International Conference on Harmonization (ICH) Guidance for Industry Q2 (R1) Validation of Analytical Procedures: Text and Methodology [18]. Linearity was also evaluated according to the procedure described by Souza and Junqueira [19].

2.4.1. Selectivity

The ultraviolet absorption spectrum, from 200 to 400 nm, of the placebo solution was obtained. In addition, three solu-
ations containing chloroquine diphosphate and the mixture of excipients in diluent, as well as three solutions containing only chloroquine diphosphate in diluent, were prepared. The absorbances of the solutions were determined and the content results were compared using analysis of variance (ANOVA).

2.4.2. Linearity

The linearity of the method was evaluated at six concentration levels, between 60% and 160% of the working concentration (12.0 µg.mL⁻¹). A standard stock solution containing 120.0 µg.mL⁻¹ of chloroquine diphosphate was prepared. Aliquots of this solution were diluted in diluent for six different concentrations, corresponding to 60%, 80%, 100%, 120%, 140% and 160% of the working concentration (7.2; 9.6; 12.0; 14.4; 16.8 and 19.2 µg.mL⁻¹). The solutions were randomly prepared and the absorbances were randomly determined.

The assumptions for the employment of the ordinary least squares method were evaluated as described by Souza and Junqueira [19]. The presence of outliers (by the Jackknife residuals test), and normality (Ryan Joiner’s test), homoscedasticity (modified Levene’s test), and independence of the residuals (Durbin-Watson test) were assessed. The regression significance and the linearity deviation were evaluated by ANOVA. The statistical significance was set at 5%.

2.4.3. Accuracy, Intraday, and Interdays Precision

Accuracy and precision were evaluated using a placebo spiking procedure. Chloroquine diphosphate was added to the mixture of excipients of the tablets to obtain three concentration levels: 60%, 100% and 160% of the working concentration. Six solutions were prepared for each concentration level, and the assay was performed on two separate days, separated by one-week interval. Precision was evaluated using the relative standard deviation (RSD) of the sample assays and accuracy was evaluated using chloroquine diphosphate recovery, which should be between 98% e 102% [20].

2.4.4. Robustness

Method robustness was assessed employing the Youden’s test [21] by means of a factorial design (Table 1). The parameters tested were ultrasound time (18 and 20 min), filtration (presence or absence) and detection wavelength (341 and 343 nm).

Chloroquine diphosphate content was determined and the effect caused by each parameter evaluated was calculated by subtracting the mean of the four values corresponding to the capital letters (nominal conditions) from the mean of the four values corresponding to the lowercase letters (changed conditions), as shown in the following equation:

\[
\text{Effect } C/c = \frac{(s+u+w+y)}{4} - \frac{(t+v+x+z)}{4}
\]

The evaluation of the effects was performed as described by César et al. [22].

2.5. Comparison of the Developed Method with the USP Chromatographic Method

In order to verify if the developed method would produce consistent and reproducible results, it was compared with the HPLC method described in the United States Pharmacopeia (USP) [11]. Chloroquine diphosphate content was determined in two batches of tablets using both methods. The results were compared using ANOVA at a significance level of 5%.

2.6. Content of Chloroquine Diphosphate in Tablets

The developed method was applied to determine chloroquine diphosphate content in six batches of the drug distributed by the Ministry of Health in northern region of Brazil. Also, the tablets were evaluated in terms of identity, hardness, friability, disintegration, dissolution and uniformity of dosage units in accordance with the Brazilian Pharmacopeia [8]. The uniformity of dosage units was evaluated by the weight variation method.

3. RESULTS AND DISCUSSIONS

3.1. Analytical Method Optimization

Initially, water was evaluated as diluent. However, the spectrophotometric method did not show adequate precision and accuracy, possibly because the pH of water was not controlled. Therefore, 0.06 M monosodium phosphate buffer pH 6.8, employed as dissolution medium for chloroquine diphosphate tablets in The International Pharmacopeia [9], was used as diluent.

The ultraviolet absorption spectrum, from 200 to 400 nm, of the standard solution and placebo solution were obtained (Fig. 2). Chloroquine showed maximum absorption wavelengths near 220 nm and in 343 nm. The placebo solution absorbs close to 220 nm, which could cause interference in

| Table 1. Factorial design for the evaluation of robustness of the analytical method. |
|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|
| **Parameter**           | **s**       | **t**       | **u**       | **v**       | **w**       | **x**       | **y**       | **z**       |
| Ultrasound time (min)   | A           | A           | A           | A           | a           | a           | a           | a           |
|                         | 20          | 20          | 20          | 20          | 18          | 18          | 18          | 18          |
| Filtration              | B           | B           | b           | b           | B           | B           | b           | b           |
|                         | yes         | yes         | no          | no          | yes         | yes         | no          | no          |
| Detection wavelength    | C           | c           | C           | c           | C           | c           | C           | c           |
| (nm)                    | 343         | 341         | 343         | 343         | 341         | 343         | 343         | 341         |
the determination of chloroquine. Therefore, chloroquine content was determined at 343 nm. Although it may represent a loss in sensitivity, the selectivity was adequate.

The specific absorption coefficient of chloroquine, obtained experimentally in this study (A 1%, 1 cm = 350) was lower than that found in the literature (A 1%, 1 cm = 625 in aqueous acid) [3]. This difference may have been caused by the use of different solvents. In acidic medium, the tertiary aliphatic nitrogen and the pyridinic nitrogen of chloroquine are protonated, presenting a positive charge. The protonation of the pyridinic nitrogen favors the extension of the conjugation by resonance of the non-binding electron pair of the secondary aliphatic nitrogen with the aromatic ring. Consequently, the energy required for the electronic excitation decreases, which may cause a bathochromic and hyperchromic effect. On the other hand, at pH 6.8, employed in this study, part of the chloroquine molecules is not protonated in pyridinic nitrogen, which explains the lower specific absorption coefficient observed for chloroquine.

3.2. Analytical Method Validation

3.2.1. Selectivity

No interfering absorption bands were observed in the absorption spectrum of the placebo solution (Fig. 2). Moreover, no statistical difference (p = 0.58), in terms of chloroquine content, was found between the sample solutions with and without added excipients (Fig. 3), showing that the latter did not interfere in the quantitative analysis of chloroquine diphosphate.

3.2.2. Linearity

Three outliers were excluded, which is in accordance with the rule of excluding a maximum of 2/9 of the data [19]. The tests of normality, homoscedasticity and independency of residuals were in agreement with all of the least squares method assumptions. The residuals presented random distribution, the regression was statistically significant (p < 0.01), and no lack of adjustment to the linear model was observed (p = 0.33). The correlation coefficient was higher than 0.99, as required [17, 23]. The regression analysis data are shown in Table 2. The analytical curve and residuals plot are shown in Fig. (4).

<table>
<thead>
<tr>
<th>Regression Parameter</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>$r^2$</td>
<td>0.9998</td>
</tr>
<tr>
<td>Slope ± SE</td>
<td>0.0376 ± 0.0002</td>
</tr>
<tr>
<td>Intercept ± SE</td>
<td>0.0087 ± 0.0020</td>
</tr>
<tr>
<td>Concentration range (µg.mL$^{-1}$)</td>
<td>7.2 - 19.2</td>
</tr>
<tr>
<td>Number of points</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 2. Overview of the linearity data for chloroquine diphosphate.
3.2.3. Accuracy, Intraday, and Interdays Precision

The mean recovery for the concentration levels of 60%, 100%, and 160% are shown in Table 3. Mean recoveries are within the 98% to 102% range, which is in agreement with the limits found in the literature [20].

The RSD values were lower than 5%, as established by Brazilian legislation [17]. It is suggested in the literature that the RSD values for intraday precision should be below 2% [20]. The RSD values obtained in the interdays precision were also less than 2%.

3.2.4. Robustness and System Suitability

The results, in terms of chloroquine content, obtained in the evaluation of robustness and the effects calculated for the parameters evaluated are presented in Tables 4 and 5, respectively.

Table 4. Results obtained in the robustness test of the analytical method.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Chloroquine Content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>s</td>
<td>99.80</td>
</tr>
<tr>
<td>t</td>
<td>99.74</td>
</tr>
<tr>
<td>u</td>
<td>99.05</td>
</tr>
<tr>
<td>v</td>
<td>99.33</td>
</tr>
<tr>
<td>w</td>
<td>99.49</td>
</tr>
<tr>
<td>x</td>
<td>99.44</td>
</tr>
<tr>
<td>y</td>
<td>98.77</td>
</tr>
<tr>
<td>z</td>
<td>98.86</td>
</tr>
<tr>
<td>Average</td>
<td>99.31</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.39</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>0.39</td>
</tr>
</tbody>
</table>

Among the parameters evaluated, filtration presented the highest difference when comparing nominal and modified values (0.62%). The absence of filtration may have resulted in lower content because the presence of particles in solution may interfere with the scattering of the light incident on the samples or with the absorption of light and may cause errors in the absorbance measurements. In addition, the presence of particles may have interfered with the pipetted volume when performing the second dilution. These particles suspended in solution after dilution of the tablets powder are likely to come from the excipients of the formulation, insoluble in the diluent used.

The method was robust in terms of ultrasound time and detection wavelength, but not for filtration. Therefore, all sample solutions must be filtered after the preparation of the first solution.

3.3. Comparison of the Developed Method with the USP Method

The results obtained with the ultraviolet spectrophotometric and the HPLC methods are presented in Table 6.

The results were compared using ANOVA at a significance level of 5%. The calculated F value for batches 1 and 2 were 3.378 and 3.126, respectively, both lower than the critical F value (5.987). Therefore, there is no statistically significant difference between the developed ultraviolet spectrophotometric method and the USP method, by HPLC.

When compared to the USP method, besides being faster and cheaper, the developed method is simpler and requires less training. Due to the need to assess the quality of the antimalarial drugs available to the population, and considering that endemic areas of the disease have low economic resources, the development and validation of quick, simple and inexpensive analytical methods are necessary to guarantee the quality, efficacy and safety of the drugs.

3.4. Quality Control of Chloroquine Diphosphate Tablets

The six batches were considered approved in the tests of identification, uniformity of weight and disintegration. The results of the tests of hardness, friability, dissolution and uniformity of dosage units, performed according to Brazilian Pharmacopeia [8], and assay are presented in Table 7.

All results were within the limits recommended by the Brazilian Pharmacopeia, except for friability, in which all batches were disapproved.

Although the weight loss was lower than the targeted value, some tablets cracked during the test. The inadequate resistance of the tablets to mechanical erosion, caused by
friction that may occur during handling, packaging and transportation, may compromise the administration of the drug in the correct dose, affecting its efficacy [24].

CONCLUSION

The developed spectrophotometric method proved to be selective, linear, precise, accurate, and robust. The method was considered equivalent to the USP method for the content determination of chloroquine diphosphate in tablets. The method proved to be simple, rapid, cheap and appropriate to be employed in the quality control of chloroquine diphosphate tablets. It can be used routinely, especially where more sophisticated techniques are not available. Chloroquine tablets distributed by Brazilian Ministry of Health in the northern region of Brazil were considered adequate in all tests, except for friability.

ETHICS APPROVAL AND CONSENT TO PARTICIPE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

Table 6. Chloroquine diphosphate contents obtained in assay with the developed method (by ultraviolet spectrophotometry) and with the USP method (by HPLC).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Batch 1</th>
<th>Batch 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UV</td>
<td>HPLC</td>
</tr>
<tr>
<td>1</td>
<td>100.92%</td>
<td>102.38%</td>
</tr>
<tr>
<td>2</td>
<td>101.93%</td>
<td>103.49%</td>
</tr>
<tr>
<td>3</td>
<td>100.93%</td>
<td>101.10%</td>
</tr>
<tr>
<td>4</td>
<td>101.21%</td>
<td>102.05%</td>
</tr>
<tr>
<td>Mean (%)</td>
<td>101.25</td>
<td>102.25</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.47</td>
<td>0.99</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>0.47</td>
<td>0.96</td>
</tr>
</tbody>
</table>

HPLC: high performance liquid chromatography; UV: ultraviolet.

Table 7. Results of the quality assessment of chloroquine diphosphate tablets.

<table>
<thead>
<tr>
<th>Test/Batch</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hardness (N)</td>
<td>50.82</td>
<td>78.87</td>
<td>71.07</td>
<td>66.30</td>
<td>62.40</td>
<td>59.80</td>
</tr>
<tr>
<td>Friability (%)</td>
<td>1.72</td>
<td>0.75</td>
<td>0.30</td>
<td>0.27</td>
<td>0.75</td>
<td>0.35</td>
</tr>
<tr>
<td>Dissolution (%)</td>
<td>95.31</td>
<td>95.50</td>
<td>94.10</td>
<td>98.22</td>
<td>97.02</td>
<td>94.21</td>
</tr>
<tr>
<td>Uniformity of dosage units (AV)</td>
<td>2.01</td>
<td>2.52</td>
<td>1.66</td>
<td>1.48</td>
<td>1.71</td>
<td>1.96</td>
</tr>
<tr>
<td>Assay (%)</td>
<td>101.25</td>
<td>101.87</td>
<td>99.67</td>
<td>101.39</td>
<td>101.79</td>
<td>100.80</td>
</tr>
</tbody>
</table>

AVAILABILITY OF DATA AND MATERIALS

The data that support the findings of this study are available from the corresponding author, [Mateus Araújo Castro e Souza], upon reasonable request.

FUNDING

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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REFERENCES

An Easy and Rapid Spectrophotometric Method for Determination

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