QUALITY BY DESIGN IN THE DEVELOPMENT AND VALIDATION OF ANALYTICAL METHOD BY ULTRAVIOLET-VISIBLE SPECTROPHOTOMETRY FOR QUANTIFICATION OF HYDROXYCHLOROQUINE SULFATE

Leslie R. M. Ferraz 1*, Fabiana L. A. Santos 1, Pablo A. Ferreira 1, Ricardo T. L. Maia-Junior 1, Talita A. Rosa 1, Salvana P. M. Costa 1, Cybelly M. Melo 1, Larissa A. Rolim 2, Pedro J. Rolim-Neto 1.

ABSTRACT: Developed originally as an antimalarial agent, the hydroxychloroquine sulfate (HCQ) is often used as a slow-acting anti-rheumatic drug in treating disorders of connective tissue. The methods of quantification of HCQ sulfate, despite the undeniable advantages, have certain limitations, such as: high cost of operation and maintenance of equipment, sample processing and analysis of the results relatively complicated. However, the simplicity of the process and velocity of the spectrophotometry by absorption in the UV-Vis region corroborate the usefulness of this methodology. This work describes the application of QbD in the development, optimization and validation of an analytical method by absorption spectrophotometry in the UV-Vis region for quantification of HCQ sulfate. The method was developed from the sample preparation used in the determination of HCQ in dosage form according to the methodology described by the USP 36. Then, the method optimization was performed using a factorial design 2. The validation was performed according to the instructions recommended by the RE No. 899/03 (ANVISA/BRAZIL). The novel method has the advantages of: use only water as a diluent solution and prepare the sample by direct dilution, making it faster and more economically viable and sustainable, given the concepts of green chemistry. The method was demonstrated: linear, effective and selective, precise and accurate and robust for all parameters evaluated. The methodology was adequate to replace the recommended USP 36 because there were no significant differences between the results obtained.

INTRODUCTION: Initially developed as an antimalarial agent, hydroxychloroquine (HCQ) sulfate - chemically (7-chloro-4-[4-[(N-ethyl-N-(2-hydroxyethyl) amino] 1-methyl[butylamino]-quinoline sulfate (Figure 1) - is often used as a slow-acting antirheumatic drug in the treatment of disorders of connective tissue 3.

**Keywords:**
Hydroxychloroquine sulfate, Analytical method, Validation, Espectrophotometry UV-Vis, Quality by Design, Method development

**Correspondence to Author:**
Leslie Raphael de Moura Ferraz
Laboratório de Tecnologia dos Medicamentos – Departamento de Ciências Farmacêuticas, Universidade Federal de Pernambuco, Rua Prof. Arthur de Sá, s/n, Cidade Universitária, 50740-521 Recife - PE, Brazil.
E-mail: lrapha@hotmail.com

**FIGURE 1. CHEMICAL STRUCTURE OF THE HCQ FREE BASE WITH THE ENUMERATION OF CARBONS.**
The HCQ has been considered into international consensus guidelines as a secondary drug for inflammatory chronic diseases therapy. Thus, HCQ is administered in conjunction with other agents, resulting in clinical efficacy of diseases such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), discoid lupus (DL), sarcoidosis, Sjögren's syndrome (SS) and photosensitivity diseases.\(^\text{4,5}\)

The drug is marketed and administered to the patient as a racemate, an equimolar mixture of two enantiomers: (+)-HCQ and (-)-HCQ. However, no information is available about the possibility of stereoselective disposition and pharmacological activities of the isolated enantiomers. Therefore, the quantification of the drug salt, HCQ sulfate, is indicated for the development of analytical methods.\(^\text{2,6}\)

The ultraviolet-visible (UV-VIS) absorption spectrophotometry is a widely used technique for measurement of molecules with chromophoric groups, part of the molecule with unsaturated and covalent group, resulting in characteristic absorption in the UV (or visible) region. It is reliable, fast and easy to use. It has low operating cost and is simple enough with regard to the interpretation of results.\(^\text{7,8}\)

The spectrophotometric scan of HCQ sulfate shows five absorption maxima at the following wavelengths: 220, 234, 256, 330 and 342 nm. These values correspond to the presence of chromophoric groups in the molecule. This last and largest of them is assigned to a quinoline group.\(^\text{9,10}\)

There are no quick and simple methods that aim the immediate assay of the raw material and pharmaceutical product formulated with HCQ in official compendia or scientific literature. The only methods described are: high performance liquid chromatography (HPLC) coupled to mass spectrometry (MS) or UV-vis,\(^\text{9,11,12}\) capillary electrophoresis\(^\text{13}\) and studies on the determination of this compound in biological fluids using chiral stationary phases or derivatization reagents, which aim to enantio selective separation.\(^\text{2,14-16}\)

Despite the undeniable advantages and better results more evidenced, the use of these techniques have certain limitations, such as: the high cost of equipment, operation and maintenance, sample processing and analysis relatively slow and the need of some experience in handling the materials and processing of results. On the other hand, equipment availability, simplicity of procedures, and the speed, precision and accuracy of spectrophotometric absorption in the UV region show that this is one of the most commonly used analytical methods.\(^\text{17-19}\)

Once developed, it is important to perform the validation of a method. To validate an analytical method is to confirm by examination and provision of objective evidence that the specific requirements for an intended use are fulfilled. The objective is to evaluate whether it provides reliable results, so it can be applied routinely. The validation consists of evaluating the capacity of the analytical process to produce consistent results with precision and accuracy considered satisfactory.\(^\text{17,20}\)

The validation process provides, to the regulatory agencies, objective evidence that the methods and systems are adequate. The parameters usually involved in the validation of analytical methods are: analytical curve, linearity, limit of detection, limit of quantification, accuracy, precision and robustness.\(^\text{21-23}\)

Quality by Design (QbD) was first described by Juran & Godfrei (1998)\(^\text{24}\) and mainly applied in the automotive industry. The fundamental premise of QbD is the design quality through an optimization strategy to establish complete understanding of the response of the quality system for determining certain variables. In the analytical and scientific research area the QbD is applied mainly in the Design of Experiments (DoE). This should be used to determine the impact of various factors and their interaction. Compared with the traditional method - a factor-at-a-time - the DoE drastically reduces the number of experiments required to determine the ideal value of each factor. This is done by varying these values in parallel. This approach determines not only the main effects of each factor, but also the interactions between factors, which are often the key to major advances.\(^\text{25-27}\)

The present study describes the development and validation of novel, alternative and simplified
analytical method for the quantification of antirheumatic drug HCQ sulfate that meets the requirements of the International Conference on Harmonization and the Brazilian Health Surveillance Agency (ANVISA) under the rules of Resolution No. 899 of 2003, which shows the practicality, reliability, safety and low cost of using this method routinely in pharmaceutical industry.

MATERIALS AND METHODS:
Raw materials, reagents and equipments
For the corresponding steps to the development of the method and its validation, it was used as raw material the HCQ acquired through FAGRON do Brasil Farmacêutica Ltda, lot 0007HS4RIIX#7, and synthesized by Sanofi-Aventis industry, while the analytical standard was purchased from Sigma-Aldrich, lot #022M4749V (declared purity of 99.78% by HPLC-DAD). The reagents, all of analytical grade, were as follows: 0.1 M hydrochloric acid (Dinâmica), distilled water (pH 7.0 ± 5), 0.1 M sodium hydroxide (Vetec), hydrogen peroxide 3% (Dinâmica), dibasic calcium phosphate (Vetec), pharmaceutical talc (Talmag Manesita) and starch (Corn Products). It was used: UV-Vis Cary 300 Conc Varian, quartz cuvette of 10 mm of optical path Varian and Spectrophotometer Varian Vankel-Cary 50 and Photostability Chamber Nova Ética (model C242), which consists of the combination of UV and fluorescent lamps with emission of 30,000 lux and 13.33 w/m² per hour. High precision analytical balances (Bioprecisa) and calibrated glassware were used for all parameters.

Development of the method by UV-Vis spectrophotometry
Principle of the method
Because there is no specific monograph in Brazilian official compendia, the method was based on the identification of the test drug by UV absorption which is recommended by United States Pharmacopeia (USP). Thus, it was used as initial parameters the reading concentration of the sample solution and the diluent solution specified: 10 μg.mL⁻¹ and 0.01 M HCl, respectively.

For the preparation of the sample, it was considered also the USP assay method of HCQ sulfate by HPLC-UV. To achieve the reading concentration (10 μg.mL⁻¹) two successive dilutions should be made, which may be the source of random errors. The absorbance analysis was realized in samples solutions with spectrophotometric scans performed in the range of 200 to 700 cm⁻¹, in triplicate.

Preparation of sample solution
The samples of HCQ sulfate were analytically weighed and solubilized in 0.01 M HCl to yield a concentration of 50 μg.mL⁻¹ (stock solution). Then, it was realized a first dilution (1:20) and a second (1:5), in order to obtain a final concentration of 10 μg.mL⁻¹, in triplicate.

Preparation of the calibration curve
Similar to the procedure performed to prepare the sample solution, the analytical standard was used to obtain the stock solution of 50μg.mL⁻¹ and to dilute with 0.01 M HCl to obtain the following concentrations: 8, 9, 10, 11 and 12 mg.ml⁻¹. The control curve was prepared each day of analysis and used to calculate concentration.

Optimization of the analytical method developed
Sample preparation was demonstrated as the first critical point to guarantee security analytics: the realization of two dilutions requires a longer running time of the experiment and can result in greater number of analytical errors. In order to overcome this problem a statistical comparison was performed (by Student's t-test) between the two successive dilutions (1:20 followed by 1:5) and only one direct dilution (1:100) to reach the concentration reading (10 μg.mL⁻¹).

The remaining optimization process was proposed to evaluate the actual need of agitation by ultrasonic bath and the use of 0.01 M HCl as a diluent solution, these conditions recommended by the specific drug monograph in the American pharmacopoeia.

From the identification of the U.S. Pharmacopeia test, a comparative analysis of the assay was performed under the following conditions: with (for 15 min) and without stirring in an ultrasonic bath in order to reduce the experimental time of the analytical method and reduce operational costs due to the use of equipment. Next, it was tested the variation of the diluent solution used (0.01 M HCl and distilled water) whereas the use of water would...
enable to reduce time, reagents and analytical waste.

To achieve the lowest possible number of experiments in a simple and orderly manner, DoE and QbD concepts have been applied. With this, it was proposed a $2^2$ factorial design to evaluate the influence of a particular variable on the precision of the method. The method has two main factors (stirring and diluent solution) and, consequently, two levels are applied (with and without agitation, using water and HCl as the diluent solution, respectively). The organization of the factorial design is shown in Table 1.

**TABLE 1. ORGANIZATION OF $2^2$ FACTORIAL DESIGN.**

<table>
<thead>
<tr>
<th>Factors</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluent solution</td>
<td>Distilled water</td>
</tr>
<tr>
<td>Sonication</td>
<td>15 minutes</td>
</tr>
</tbody>
</table>

In order to evaluate the influence of the variables in the experiment, as well as the interaction between them, the Pareto chart was selected as a tool. All results were expressed in the confidence interval of 95%.

**Validation of the analytical method developed**

Once developed, the following parameters were evaluated: selectivity, robustness, linearity, limit of detection, limit of quantification, precision and accuracy. These parameters were evaluated according to the standards set by ICH 26, 27 and the Resolution No. 899 of 2003 (ANVISA) 21, since this work has been classified as an analytical method for the quantitative determination. The confidence level of the results was observed for the following treatments: coefficient of variation (CV %) less than 5% and statistical analysis using Student's t-test, One-Way and Two-Way ANOVA test when applicable.

**Specificity / Selectivity**

The selectivity and/or specificity of a method ensures that the measurement of the sample of interest is not affected by the presence of metabolites, degradation products, co-administered drugs or adjuvants used in formulations 28. To evaluate the selectivity, a preliminary study of forced degradation of the drug was performed. For this, the samples were subjected to the following stress conditions: acid and basic hydrolysis (1M HCl and 1M NaOH, respectively), oxidation (hydrogen peroxide 5%) - in the ratio 3:1 degradative solution: sample - and photolysis (45 ± 5°C/15 min). The solutions were maintained at room temperature and analyzed after 72 hours. By the method developed and compared with analysis by HPLC-DAD according to the U.S. Pharmacopoeia monograph 9.

The specificity of the method was evaluated regarding the contamination of the sample with excipient ingredients of the reference medicine, a solid dosage form (tablet) Plaquenil®, which are: starch, talc and dibasic calcium phosphate. All physical mixtures were prepared in 2:1 drug: excipient and the samples were weighed with respect to the concentration of HCQ sulfate.

**Robustness**

To evaluate the performance of the method compared to small and deliberate modifications, the following parameters were alternated and applied to sample preparation and/or analytical method: influence of light during sample preparation through the presence and absence of cold white light (for no more than 25 minutes); stability of the sample at times 0, 2 and 4 hours and reading wavelengths: 341, 342 and 343 nm, mimicking the possible lack of calibration of the equipment 28.

**Linearity**

To examine the ability of the method to demonstrate the results obtained are directly proportional to the analyte concentration of the sample, authentic three linear regression curves were performed, using the adjustment data by the method of least squares of the midpoints, and five different concentrations 8, 9, 10, 11 and 12 μg.mL$^{-1}$. The average variation of the study was to range from 80% to 120% of the mean concentration of HCQ sulfate.

**Limit of detection (LOD) and limit of quantification (LOQ)**

The LOD is the lowest concentration of the substance under examination which can be detected with a certain limit of reliability. It corresponds to the concentration that produces a sign three times greater than the average noise level measured with
the control or blank solution. The LOD was calculated from the ratio between the standard deviation (SD) of the linear coefficients of the three calibration curves of the linearity tests and the average of the angle coefficients (a) of the respective curves, multiplied by 3. This same principle was used to calculate the LOQ. However, the ratio was found 10 times the baseline. The two equations used are outlined below.

Equation 1: Limit of detection = \( \frac{3SD}{a} \)

Equation 2: Limit of quantification = \( \frac{10SD}{a} \)

**Precision**

This parameter corresponds to the criteria of repeatability, intermediate precision and reproducibility. In this work, three levels were evaluated. The repeatability (intra-assay precision) corresponds to the statistical proximity between the results of the same group of samples, so it was realized by the same analyst and same equipment. The intermediate precision (inter-assay precision) represents the agreement of the results obtained in the same laboratory, but performed on different days and by different analysts. Whereas, reproducibility evaluates the concordance of results obtained in different laboratories and, therefore, by different analysts. The repeatability was verified by six authentic determinations to 100% of the concentration of the test. The same was used for the intermediate precision, but it was performed on different days by different analysts. Reproducibility was assessed in two different laboratories: Laboratório de Tecnologia dos Medicamentos (LTM) and Núcleo de Controle de Qualidade de Medicamentos e Correlatos (NCQMC), both laboratories are from UFPE.

**Accuracy**

The statistical similarity of the results in comparison to standard theoretical values was analyzed from samples of HCQ sulfate, raw materials and analytical standard at concentrations of 50, 100 and 150% of the reading concentration (10 \( \mu \)g.mL\(^{-1} \)), in triplicate.

**Application of the method**

The validated methodology was tested in a batch of raw material HCQ sulfate (FAGRON\textsuperscript{®} Pharmaceuticals in Brazil Ltda, Lot 0007HS4RJCX #7) and a brand of processed tablets: the reference drug (Plaquenol\textsuperscript{®}, Sanofi-Aventis Lot 04618) recognized by ANVISA (2013). The results were compared, in a similar manner, by the UV assay of the raw material and of the analysis of the tablets, both described in U.S. Pharmacopeia USP 36, and it consists in a variation of the method for analysis of raw materials. In order to assess the differences between the mean of the content obtained, it was performed statistical analysis by Student's t test.

**RESULTS AND DISCUSSION**

**Method development**

The spectrophotometric scan of HCQ sulfate showed five absorption maxima at the following wavelengths: 220, 234, 256, 330 and 342 nm (Figure 2). These values correspond to the presence of chromophoric groups present in the molecule. This last and largest of them is assigned to a quinoline group and, therefore, was selected as the wavelength to the analysis of samples.

![Absorption Spectra](image)

**FIGURE 2. ABSORPTION SPECTRA IN THE ULTRAVIOLET REGION OF HCQ SULFATE.**

**Optimization of the method**

**Preparation of the sample**

The modification of the sample preparation consists in performing just one dilution to achieve the concentration of reading showed to be satisfactory. The Student’s t-test was used to show if there were differences between the averages obtained. In this case, the null hypothesis (H\textsubscript{0}) must be accepted, since the \( t_{cal} \) was lower than \( t_{tab} \) with confidence interval of 95%, and 5 degrees of freedom (Table 2). This implies that the averages are statistically
equal and that only one dilution can be used for sample preparation.

TABLE 2. COMPARISON BETWEEN THE RESULTS OBTAINED OF ANALYZES WITH DIFFERENT FORMS OF SAMPLE PREPARATION AND STATISTICAL ANALYSIS ASSUMING DIFFERENT VARIANCES

<table>
<thead>
<tr>
<th>Preparation of the sample</th>
<th>Samples – Concentration (µg/mL)</th>
<th>Mean (µg/mL)</th>
<th>Standard Deviation</th>
<th>t_{cal}</th>
<th>t_{0.95,5}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Dilution</td>
<td>10.90</td>
<td>10.96</td>
<td>10.94</td>
<td>10.93</td>
<td>0.033</td>
</tr>
<tr>
<td>2 Dilutions</td>
<td>10.49</td>
<td>10.53</td>
<td>10.57</td>
<td>10.53</td>
<td>0.043</td>
</tr>
</tbody>
</table>

In fact, there was a reduction in the time taken to make the sample (from ± 40 min to ± 15 min). It was also possible to avoid inserting new random errors arising from more than one dilution. This may be due to more accuracy between samples prepared from only one dilution.

Factorial design $2^2$ to optimization methods

For the results of the factorial analysis of the influence of diluent solution and agitation in ultrasonic bath, the results described in Table 3 and 4 were obtained.

TABLE 3. MATRIX OF FACTORIAL DESIGN $2^2$.

<table>
<thead>
<tr>
<th>Essay</th>
<th>Results</th>
<th>Mean</th>
<th>Variance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>0.455</td>
<td>0.441</td>
<td>0.455</td>
</tr>
<tr>
<td>2</td>
<td>0.427</td>
<td>0.417</td>
<td>0.421</td>
</tr>
<tr>
<td>3</td>
<td>0.452</td>
<td>0.462</td>
<td>0.472</td>
</tr>
<tr>
<td>4</td>
<td>0.456</td>
<td>0.443</td>
<td>0.442</td>
</tr>
</tbody>
</table>

TABLE 4. INFLUENCE FACTORS AND THE INTERACTION BETWEEN THEM.

<table>
<thead>
<tr>
<th>Levels</th>
<th>Influence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluent Solution</td>
<td>-0.450</td>
</tr>
<tr>
<td>Sonication</td>
<td>-0.450</td>
</tr>
<tr>
<td>Interaction</td>
<td>-0.462</td>
</tr>
</tbody>
</table>

The Pareto chart (Figure 3) shows the values of the estimated effects, which allows to verify whether they are statistically significant. Here, the effect is as significant as the rightmost of the dashed line, because it corresponds to the limit of significance, calculated for the confidence interval of 95%. According to the graph, distilled water as the diluent solution is the factor that has the greatest influence on the precision of the method. Another important information obtained from the graph is the significant influence of the absence of sonication, as shown in the graph, it occurs in a reverse form, since it corresponds to the negative factor. The value to the left of the limit of significance shows that there is no significant influence when the interaction between the factors is evaluated.

The results obtained show the advantages of the method. The lack of need to stir generates electric energy savings and allows rapid sample preparation. Thus, the method becomes faster and more satisfactory. Changing the diluent solution of 0.01 M HCl to distilled water satisfies some principles of green chemistry proposed by Anastas & Warner (1989) and Machado (2012), such as: the principles 1 and 2 (waste prevention and more inherent safety regarding accidents, respectively), the principle 22 (quantifiable and minimizing the use of “utilities” such as water and...
electric energy) and principle 24 (monitoring, record and waste minimization). Thus, we showed that the method is adequate and fits to the concepts of sustainability.

**Validation of the method**

The Figure 4 shows the overlaid spectra of HCQ sulfate, the sample of HCQ sulfate contaminated with excipients of the solid formulation and of the pharmaceutical form itself (pill - Plaquenol). The scans showed characteristic peaks only the maximum absorption of HCQ sulfate (220, 234, 256, 330 and 342 nm), confirming that the method is specific for the detection and quantification of the drug, even if it is in the presence of other compounds, such as pharmaceutical adjuvants.

![Figure 4. Spectrophotometric scans of HCQ isolated, contaminated with excipients and tablet dosage form Plaquenol.](image)

To determine the selectivity of the method, the submission of samples to stress conditions was proposed in order to show that the degradation products do not interfere in the method. As expected, these conditions caused significant reduction of the percentage of the content of the analyzed samples, indicating that degradation products were formed and these, in turn, do not influence the dosing of the drug, since the absorbance obtained from each sample is virtually equal to that calculated in the theoretical analysis when compared to the analysis performed by HPLC-DAD according to the U.S. pharmacopoeia monograph. Thus, the method proved to be sensitive enough to prevent that the presence of similar structures derived from the degradation were able to interfere with the quantification of the HCQ sulfate.

**TABLE 5. RESULTS OBTAINED FOR THE ANALYSIS OF THE SELECTIVITY PARAMETER.**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Sample concentration (µg.mL⁻¹)</th>
<th>Content (%)</th>
<th>Degradated Content (%)</th>
<th>Expected Absorbance</th>
<th>Obtained Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M NaOH</td>
<td>10</td>
<td>21.82</td>
<td>78.18</td>
<td>0.085</td>
<td>0.087</td>
</tr>
<tr>
<td>1M HCl</td>
<td>10</td>
<td>34.24</td>
<td>65.76</td>
<td>0.135</td>
<td>0.137</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>10</td>
<td>29.11</td>
<td>70.89</td>
<td>0.114</td>
<td>0.116</td>
</tr>
<tr>
<td>Photolysis</td>
<td>10</td>
<td>88.49</td>
<td>11.51</td>
<td>0.353</td>
<td>0.354</td>
</tr>
</tbody>
</table>

The results obtained from the points of three authentic curves of samples of HCQ sulfate are shown in Figure 5a. By linear regression analysis by the least squares method, it was possible to determine the value of the correlation coefficient $r^2 = 0.99925$. This value indicates that 99.9% of the total variation around the mean is explained by the regression, demonstrating that there is a linear correlation between the two variables (absorbance and concentration), since the parameters are within the prescribed thresholds ($r^2 \geq 0.99$) ²⁷. The scatter plot shown in Figure 5b shows that there was no lack of adjustment in the method. Therefore, there was a little presence of random errors, not showing any systematic error in the
analysis, which can be observed by heteroscedastic distribution of the points. Other relevant information presented by the same graph is that, at least, one point touches the line. This means that one of the values obtained in the analysis corresponds faithfully to the reference values. 

The values of LOD and LOQ were 0.38 and 1.27 μg.mL⁻¹, respectively. These values indicate that the method is very sensitive with respect to the detection and quantification of the drug without major interference from the equipment.

The method proved to be precise in three levels: repeatability, intermediate precision and reproducibility. Regarding repeatability, the results were satisfactory, with an average of 10.94 ± 0.13 μg.mL⁻¹ with a coefficient of variance of 1.15%, which was below the maximum value recommended by ANVISA (5%) as for the intermediate precision, it was evidenced statistically by two-way ANOVA that the method was precise for analyzes performed by different analysts on the same day as either in different days. This is due to the fact that the F values calculated were always lower than the tabulated F (critical). These same concepts and treatment were used to assess the precision of the method in relation to the parameter of reproducibility.

The accuracy was assessed by comparing each of the theoretical and actual concentrations, using Student’s t-test assuming equal variances. The results of the foregoing parameters are shown in Table 7.
TABLE 7. RESULTS OBTAINED FOR THE ANALYSIS OF THE ACCURACY PARAMETER AND STATISTICAL ANALYSIS STUDENT'S T-TEST ASSUMING EQUAL VARIANCES.

<table>
<thead>
<tr>
<th>Concentration (µg.mL⁻¹)</th>
<th>Results</th>
<th>Mean (µg.mL⁻¹) ± DP</th>
<th>Student’s t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>5,07</td>
<td>4,92</td>
<td>5,05</td>
</tr>
<tr>
<td>10</td>
<td>9,96</td>
<td>9,94</td>
<td>9,96</td>
</tr>
<tr>
<td>15</td>
<td>14,98</td>
<td>15,13</td>
<td>15,05</td>
</tr>
</tbody>
</table>

As to robustness, the method has considerable resistance to small and deliberate modifications to the test conditions, since all F-calculated were smaller than F tabulated when using one-way ANOVA. The analysis results are summarized in Table 8.

TABLE 8. RESULTS OBTAINED FOR THE ANALYSIS OF THE ROBUSTNESS PARAMETER AND STATISTICAL ANALYSIS USING ONE-WAY ANOVA.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Variables</th>
<th>Time (hour)</th>
<th>Mean (µg.mL⁻¹) ± DP</th>
<th>One-Way ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luminosity</td>
<td>Absence of light</td>
<td>-</td>
<td>10,57 ± 0,08</td>
<td>F calculated: 0,13</td>
</tr>
<tr>
<td></td>
<td>Presence of light</td>
<td>-</td>
<td>10,58 ± 0,02</td>
<td>F tabulated: 7,70</td>
</tr>
<tr>
<td>Estability</td>
<td>-</td>
<td>0</td>
<td>10,51 ± 0,07</td>
<td>F calculated: 1,22</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>2</td>
<td>10,55 ± 0,07</td>
<td>F tabulated: 7,70</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>4</td>
<td>10,57 ± 0,06</td>
<td></td>
</tr>
<tr>
<td>Wave length</td>
<td>341 nm</td>
<td>-</td>
<td>10,44 ±0,06</td>
<td>F calculated: 2,12</td>
</tr>
<tr>
<td></td>
<td>342 nm</td>
<td>-</td>
<td>10,62 ± 0,13</td>
<td>F tabulated: 7,70</td>
</tr>
<tr>
<td></td>
<td>343 nm</td>
<td>-</td>
<td>10,52 ± 0,08</td>
<td></td>
</tr>
</tbody>
</table>

Aplication of the method

The results presented in Table 9 show that, based on the values obtained in the Student’s t-test, there are no significant differences between the proposed method and the official method of the U.S. Pharmacopeia, both raw material and dosage form, with confidence interval of 95% of significance. In assessing content of HCQ by the method proposed here in the commercial raw material and industrial tablets, mean values were 99.38% and 102.24%, respectively.

According to the U.S. Pharmacopoeia, the HCQ sulfate must demonstrate content between 98 and 102% for the raw material and from 93 to 107% for the dosage form. Thus, it was observed that both materials are within the prescribed limits.

TABLE 9. RESULTS OBTAINED FOR THE CONTENT OF HCQ IN RAW MATERIAL AND TABLETS AND STATISTICAL TREATMENT BY STUDENT’S T-TEST.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Proposed Method</th>
<th>Official method (USP 32)</th>
<th>t_cal</th>
<th>t_tab</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw material</td>
<td>99,38%</td>
<td>98,45%</td>
<td>0,0355</td>
<td>4,3027</td>
</tr>
<tr>
<td>Tablet</td>
<td>102,24%</td>
<td>104,01%</td>
<td>3,391</td>
<td>4,3027</td>
</tr>
</tbody>
</table>

CONCLUSION: The proposed method has the advantages of: using only water as a diluent solution and preparing the sample by direct dilution, making it faster, more economically viable and sustainable, given the concepts of green chemistry.

The optimized method was validated according to the criteria of the Resolution No 899/03 of ANVISA and the ICH and demonstrated to be: linear, effective and selective, precise and accurate and robust for all parameters evaluated. Furthermore, the methodology was adequate to replace the official one recommended by the USP 36, because there were no significant differences between the results obtained by both techniques. It was concluded that the use of spectrophotometric techniques with UV absorption for the quantification of HCQ sulfate has fundamental role for quality control testing and essay of the drug and
dosage form, proving to be a reliable and safe technique for this purpose.

ACKNOWLEDGEMENTS: To the Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco (FACEPE) the granting of financial assistance and the NCQMC, whose infrastructure was used in part of the elaboration of this work.

REFERENCES:

29. Lloyd DK and Bergum, J: Chapter 3 – Application of quality by design (QbD) to the development and validation of analytical methods. Specification of Drug Substances

E-ISSN: 0975-8232; P-ISSN: 2320-5148

How to cite this article: