Development and validation of analytical methodology for quantification of propranolol hydrochloride in a multiparticulate biphasic system by UV-vis spectrophotometry

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ABSTRACT. Hypertension is one of the most important mortality risks in the world and is considered the leading cause of deaths associated with cardiovascular disease. In an attempt to improve the treatments and apply them to a large number of disorders, a propranolol hydrochloride (Prop) based medicine it was developed in our laboratory, employing a multiparticulate system which operates in a biphasic manner, one portion of immediate and other of modified of release. This study aimed at developing and validating a suitable method to quantify this new version of the drug using UV spectrophotometry, following what is recommended in RE 899/2003. The results showed that the method employed was linear, accurate, precise and robust in the range of 0.8 to 96.0 μg mL⁻¹, which allows it for routine use in quality control laboratories.

Keywords: biphasic release, multiparticulate system, pellets.

Desenvolvimento e validação de metodologia analítica para quantificação de cloridrato de propranolol em sistema multiparticulado bifásico por espectrofotometria ultravioleta

RESUMO. A hipertensão é um dos mais importantes riscos de mortalidade no mundo, sendo considerada a principal causa de mortes associadas a doenças cardiovasculares. Em uma tentativa de obter tratamentos mais eficazes e aplicados a um grande número de desordens, foi desenvolvido, em nosso laboratório, um medicamento à base de cloridrato de propanolol (Prop), empregando um sistema multiparticulado que agisse de forma bifásica, sendo uma fração de liberação imediata e outra de liberação prolongada. Este trabalho teve como objetivo desenvolver e validar um método direcionado para a quantificação dessa nova versão do medicamento, empregando espectrofotometria UV e seguindo o recomendado na RE 899/2003. O método proposto foi linear, exato, preciso e robusto na faixa de 0,8 a 96 μg mL⁻¹, o que permite sua utilização na rotina de um laboratório de controle de qualidade.

Palavras-chave: liberação bifásica, sistema multiparticulado, péletes.

Introduction

Hypertension is a major factor risk for overall mortality, affecting one in three adults of 25 years or more and with an overall prevalence of 22% in adults over 18 years. Considered responsible for most of heart diseases and strokes, it accounts for about 9.4 million deaths related to cardiovascular disease every year (World Health Organization [WHO], 2015a; 2015b).

Complications related to cardiovascular diseases occur predominantly at the early morning hours, since the blood pressure is higher. This fact relates to the circadian rhythm, which causes physiological and biochemical fluctuations in the human body along the day (Ohdo, 2010; Lin & Kawashima, 2012; Verdecchia et al., 2012; Dallmann, Brown, & Gachon, 2014).

In an attempt to obtain a more effective therapeutic system for the treatments of hypertension that exists in the pharmaceutical market and, considering the physiological changes of the circadian rhythm, a biphasic multiparticulate system it was developed in our laboratory containing the drug propranolol hydrochloride (Prop), composed of a fraction of immediate release and other of modified release. This system, directed to the treatment of hypertension, aims at minimizing pressure peaks that occur in the morning.
maintaining therapeutic levels of the drug all over the day (Maggi, Machiste, Torre, & Conte, 1999; Londhe, Gattani, & Surana, 2010; Jha, Rahman, & Rahman, 2011).

The Prop drug, which is in the class of nonselective β-adrenergic blockers, is widely prescribed for treating cardiovascular diseases such as angina, arrhythmias, hypertension, and myocardial infarction. In Brazil, medications containing this drug belong to the National List of Essential Medicines (Brasil, 2013), representing drug access and wide consumption by the population. With regard to the pharmacokinetics of the immediate-release Prop, the drug has a peak plasma concentration of 1 to 1.5 hours after administration and a half-life of 4 to 6 hours. This rapid absorption time and half-life, culminating in the need for multiple administrations during the day, which create discomfort to the patients, thus representing an opportunity for the development of drugs with modified release systems (Goodman & Gilman, 2001; Bortolotto & Consolim-Colombo, 2009).

Concern about the quality of medicines available in the market has intensified over the years, since parameters such as quality and efficiency directly influence the treatment of diseases and consequently the health of the patient. Therefore, regulatory agencies have been increasing the requirements during the registration of new drugs, so that the methods of analyses used are suitable (Brasil, 2003; Ribani, Bottoli, Collins, Jardim, & Melo, 2004; International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use [ICH], 2005; Santana et al., 2009; Grillo et al., 2009; Nascimento, Rosa, Nishijo, & Aversi-Ferreira, 2011).

Drug quantification is extremely important during the development stage and quality assurance of drugs, but when it comes to new delivery systems, analytical methods are not available in official compendia and should therefore be developed specifically for the system in question (Barros, 2002).

The analytical methodology provides for the preparation of the sample for the measurement of active ingredient, and the procedures involved in the preparation and also in the analysis must be established during the analytical development, later to be submitted to method validation. The optimization of these parameters is necessary in order to obtain the best analytical results linked to analysis with smaller time/cost ratio. This process, when properly defined and documented, provides evidence for regulatory agencies and ensures that the proposed method is reproducible, efficient and suitable for use in laboratory routine (Ribani et al., 2004; ICH, 2005; La Roca, Sobrinho, Nunes, & Neto, 2007).

A method is considered valid when its characteristics are in accordance with the prerequisites set for each methodological purpose or every time a methodology is developed or suffers adaptations. Generally, the criteria accuracy, precision, detection and quantification limits, robustness and specificity are discussed (Brasil, 2003; Brito, Amarante Jr., Polese, & Ribeiro, 2003; Valentini, Sommer, & Matioli, 2004).

During the stage of development of an analytical method, there is great interest in fast and efficient methods capable of providing accurate results for the quantification of drugs that can be deployed easily in the laboratory routine, and that above all, require little investment. An example with these characteristics is a technique of absorption spectrophotometry in the ultraviolet-visible region (UV-Vis) a simple, fast and inexpensive technique, widely used in quality control laboratories and easy of handling (Komarova et al., 2009; Alves et al., 2010; Spagnol, Oliveira, Isaac, Corrêa, & Salgado, 2015).

Thus, this paper proposes the development and validation of an analytical method for assay analysis and dissolution of Prop contained in a multiparticulate system of modified release, by absorption spectrophotometry, ultraviolet-visible (UV-Vis), to be used in quality control analysis of a new drug delivery system.

Material and methods

Chemical substance reference and reagents

The chemical substance reference (CSR) used was propranolol hydrochloride (Prop) - The United States Pharmacopeia (Lot I1G348) with 99.90% content.

The reagents methanol and hydrochloric acid (HCl), all of analytical grade, were purchased from Scharlau and Cromoline, respectively.

Samples

Capsules with 80 mg of propranolol hydrochloride, containing 40 mg of immediate release pellets and 40 mg of modified release pellets, were evaluated. Placebos were also prepared, containing the same amounts described in the formula above except the active ingredient.

Developing of the analytical methodology

Dosing

To determine the Prop content in the samples, the content of 20 capsules were used (approximately
7.28 g). The pellets were ground with the aid of a mortar and pestle and a mass equivalent to 25 mg of Prop was weighed and transferred to a 25 mL volumetric flask. 2 mL of methanol were added and the flask placed in the ultrasonic bath for 40 min. Subsequently 12.5 mL of methanol were added and the flask was placed in the ultrasonic bath for additional 20 min. Then, the volumetric flask volume was completed with the same solvent. From this solution an aliquot of 2 mL was transferred to a 25 mL volumetric flask and the volume completed with aqueous 0.01 mol L⁻¹ HCl. The obtained final concentration of Prop was 80 μg mL⁻¹. All solutions were filtered through a quantitative cellulose filter. The solution was analyzed by UV-Vis spectrophotometer at 289 nm using 0.01 mol L⁻¹ HCl for zero adjustment.

**In vitro dissolution test**

For the dissolution test, a dissolutor USP 1, 2, 5, 6 SOTAX® AT 7smart semiautomatic UV-Vis online was used, and the quantification was performed using the spectrophotometric method in the UV-Vis region, as determined in development methodology. The following conditions were recommended: solution used as the dissolution medium (1000 mL of 0.01 mol L⁻¹ HCl), temperature (37 ± 0.5°C), apparatus (basket, 100 rpm), six tanks and the test time (24 hours). The final sample concentration was 80 μg mL⁻¹, concentration used in the same content test. The samples were quantified using an online reading system with UV-Vis detector, using a wavelength of 289 nm.

**Validation of the analytical method using UV-vis spectrophotometry**

The validation of the methodology was carried out by following the recommendations for validation of analytical and bioanalytical methods, described in Resolution N. 899 of 29 May 2003 (Brasil, 2003), evaluating the parameters established for categories I (dosing) and III (dissolution). For spectrophotometric determinations, a Varian Cary® 50 spectrophotometer was used.

Although the Resolution N. 899/2003 established the performing of inter-laboratory assays, the high cost of these tests did not allow their application.

**Preparation of the standard solution**

10 mg of Prop were transferred to a 25 mL volumetric flask. 2 mL of methanol were added and the mixture was placed in ultrasonic bath for 10 min to allow complete solubilization of the active, and the volume was completed with 0.01 mol L⁻¹ HCl. From this solution, 2 mL were transferred to a 10 mL volumetric flask and the volume was completed with the same solvent, resulting in a solution with Prop concentration of 80 μg mL⁻¹.

**Specificity and selectivity**

For the definition of Prop maximum absorption wavelength (λ_{max}), an UV-Vis scan was performed ranging from 200 to 400 nm. The specificity/selectivity of the method was determined by evaluation of the interference of placebo on the quantification of the drug. The placebo consisted of all excipients and coatings, including the capsules without active ingredient. This evaluation was performed by weighing three triplicate placebo mixture samples, which were then dispersed in 0.01 mol L⁻¹ HCl. The first triplicate corresponded to 100% of the amount of placebo and the following two formulations presented an increase of 25 and 50% compared to the first placebo. Sample extractions were performed at 37°C and the placebo interference was determined in comparison with the Prop standard solution (The United States Pharmacopeia [USP], 2015).

**Linearity**

To determine the linearity, a calibration curve was plotted at Prop concentrations of 0.8, 4.0, 8.0, 16.0, 32.0, 48.0, 64.0, 80.0 and 96.0 μg mL⁻¹ and was evaluated in triplicate. These solutions were prepared by taking aliquots from a stock solution of 400 μg mL⁻¹. The linear fit was obtained by employing the least squares method and was evaluated by an analysis of variance (ANOVA).

**Range**

Range is the interval between the upper and lower limits of quantification of the analytical method. It is established by confirming that the method has precision, adequate accuracy and linearity when applied to samples containing quantities of substances within the specified range.

**Precision**

To determine the repeatability (intra-run precision), 12 determinations for the content were performed at a concentration of 80 μg mL⁻¹, 6 for the content method and 6 for dissolution one. For intermediate precision (inter-run precision), replicates of these concentrations were prepared and analyzed by different analysts in different days.

**Accuracy**

The accuracy test was carried out by triplicate testing of the samples at concentrations of 0.8, 4.0,
48.0 and 96.0 μg mL⁻¹. The accuracy is expressed as a ratio of the experimentally determined concentration, and the corresponding theoretical concentration, being expressed according to Equation 1:

\[
\text{Accuracy} = \frac{c_{\text{exp}}}{c_t} \times 100
\]

where:
- \( c_{\text{exp}} \): experimental average concentration;
- \( c_t \): theoretical concentration.

**Robustness**

The robustness of the method was performed in triplicate, using solutions of 80.0 μg mL⁻¹ of Prop, with small variations in the analytical conditions of the method. The changed parameters were: wavelength (284, 289 and 294 nm), sonication time (5, 10 and 15 min) and different brands of HCl solvent (Brasil, 2003; ICH, 2005; Borba, Riekes, Pereira, & Stulzer, 2013).

**Drug stability in solution**

The evaluation of the drug stability in solution was assessed in three points: in the stock solution and in the content and dissolution samples. The first two were evaluated at room temperature and the last one at 37°C. Responses were evaluated at 0, 12, 24 and 36 hours, and the acceptable range of stability of the solutions was from 98 to 102% compared with the initial analyses of solutions (USP, 2015).

**Statistical analysis**

The statistical analysis of the data was performed by one-factor ANOVA, where the results were considered significant when the probability was less than 5% (p < 0.05, confidence interval 95%). Student test t with a significance level \( \alpha = 0.05 \) (95% confidence interval) was applied, using the Microsoft Office Excel® software, version 2007.

**Results and discussion**

**Validation of analytical method by spectrophotometry in the UV range**

The acquired UV-Vis spectrum of Prop in HCl 0.01 mol L⁻¹ solution (Figure 1 line A) showed a \( \lambda_{\text{max}} \) at 289 nm. The placebo UV-vis spectrum in the same media and in the same range (Figure 1 line B) showed no overlapping peaks with Prop. The values obtained for the interference of the placebo were 0.45, 0.22 and 0.26% for placebo concentrations of 100, 125 and 150%, respectively, thus confirming the absence of interfering excipients used in formulation for this same wavelength and the specificity of the method, since they do not exceed the specification of 2.0%. The ANOVA demonstrated that there were no significant differences between analyses with different concentrations of placebo, and calculated \( F \) (Fcal = 2.404) and tabulated \( F \) (Ft = 5.143) at p < 0.05.

**Figure 1.** Absorption spectra in the ultraviolet region of the Prop standard solution (A) and placebo (B).

The linearity is the ability of an analytical method to demonstrate that the results obtained are directly proportional to the concentration of analyte in the sample, within a specified range. Thus, it was obtained by the analytical curve of Prop (Figure 2) for examining nine different concentrations of the sample, determined previously, according to the dissolution profile data (Brasil, 2003; Brito et al., 2003).

**Figure 2.** Plot of the calibration curve and equations of the standard Prop solution, obtained by the spectrophotometric method in the ultraviolet region.

According to the RE 899/2003 (Brasil, 2003), the acceptable minimum criterion of the correlation coefficient (r) should be 0.99.

Thus the obtained analytical curve (Figure 2) was linear in the range of 0.8 to 96.0 μg mL⁻¹, since...
the obtained results were proportional to the concentration of the analyte (Table 1).

Table 1. Data for linearity method.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity range</td>
<td>0.8 – 96.0 μg mL⁻¹</td>
</tr>
<tr>
<td>Equation: y = ax + b</td>
<td>y = 0.01967x + 0.00452</td>
</tr>
<tr>
<td>Correlation coefficient (r)</td>
<td>0.9999</td>
</tr>
</tbody>
</table>

Although the value of the correlation coefficient is within the specified value, it is necessary to carefully evaluate the residues of the linear regression (Figure 3) to ensure the adequacy of the linear fit to the calibration curve. It was noted that residues followed uniform distribution, constant variance (homocedasticity) and the absence of atypical samples, this way confirming the proper fitting method (Ribeiro & Ferreira, 2008; Gomes & Souza, 2010).

Figure 3. Plot of the residues.

Precision evaluates the closeness of the obtained results in a series of measurements of a multiple sampling of the same sample. Repeatability was assessed on one day and by the same analyst, and the average values found in these tests were (99.54 ± 0.96)% for dosing assays, and (99.52 ± 0.73)% for dissolution measurements, presenting no significant differences between them with calculated F (Fc = 0.001) and tabulated F (Ft = 4.964) for p < 0.05. The intermediate precision was determined on two different days by two different analysts. The values of these determinations are presented in Table 2, and the average relative standard deviation did not exceed the value of 5%. ANOVA showed that there were no significant differences between the tests carried out by analysts 1 and 2 during the study period, according to calculated F (Fc = 1.504) and tabular F (Fc = 2.249) at p < 0.05. Thus, the method proved to be accurate in accordance with this experiment (Brasil, 2003; ICH, 2005).

The accuracy is the closeness of the results obtained in relation to the true value. In the present study, the degree of recovery was determined for the concentrations of 1, 5, 60 and 120%. (Brasil, 2003). The limit of acceptable recovery levels for pharmaceutical products is between 95 - 105% (USP, 2015). The test conducted for all concentrations demonstrated the accuracy of the method, since the results obtained (Table 3) were within specified limits. From the Student t test, we could confirm that the recovery value found is statistically equal to 100%, with significance level α = 0.05, calculated T (Tc = -0.952) and tabulated T (Tt = 1.717).

Table 2. Results obtained in the intermediate precision of analysis by spectrophotometric method for the determination and evaluation of dissolution of biphasic release Prop capsules.

<table>
<thead>
<tr>
<th>Methodology</th>
<th>Analyst</th>
<th>Concentration Day 1 (%)</th>
<th>Concentration Day 2 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dosage</td>
<td>1</td>
<td>99.83 ± 0.66</td>
<td>99.33 ± 0.30</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>100.02 ± 1.36</td>
<td>99.17 ± 0.43</td>
</tr>
<tr>
<td>Dissolution</td>
<td>1</td>
<td>98.81 ± 1.28</td>
<td>99.23 ± 0.31</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>99.92 ± 1.17</td>
<td>99.08 ± 0.80</td>
</tr>
</tbody>
</table>

*Mean ± RSD (n = 6); RSD: Relative Standard Deviation.

Table 3. Results obtained by the spectrophotometric method of accurate analysis for biphasic release Prop capsules.

<table>
<thead>
<tr>
<th>Theoretical concentration (μg mL⁻¹)</th>
<th>Average experimental concentration (μg mL⁻¹)</th>
<th>Recovery (%)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.80</td>
<td>0.80</td>
<td>100.55</td>
<td>1.90</td>
</tr>
<tr>
<td>4.00</td>
<td>3.98</td>
<td>99.40</td>
<td>1.73</td>
</tr>
<tr>
<td>48.00</td>
<td>48.58</td>
<td>101.21</td>
<td>0.74</td>
</tr>
<tr>
<td>96.00</td>
<td>96.29</td>
<td>100.31</td>
<td>0.28</td>
</tr>
</tbody>
</table>

*Mean (n = 3); RSD: Relative Standard Deviation.

The robustness of an analytical method is a measure of its ability to withstand small changes in analytical parameters to assess the susceptibility of the method to variations. The modified analytical parameters (Table 4) did not show significant changes in content of the samples and is therefore considered a robust method with respect to the change of the brand of the HCl solvent, sonication time and wavelength. The ANOVA statistical analysis demonstrated that there are no significant differences between the analyses with different parameters, and calculated F (Fc = 1.674) and tabulated F (Fc = 5.143) at p < 0.05.

Table 4. Data for the robustness method employed.

<table>
<thead>
<tr>
<th>Factors</th>
<th>Sample absorbance (%)</th>
<th>Drug concentration (%)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent brand</td>
<td>1.558</td>
<td>99.17</td>
<td>2.18</td>
</tr>
<tr>
<td>C</td>
<td>1.565</td>
<td>98.87</td>
<td>0.59</td>
</tr>
<tr>
<td>Sonication time</td>
<td>5.158</td>
<td>99.58</td>
<td>0.46</td>
</tr>
<tr>
<td>(mm)</td>
<td>10</td>
<td>100.77</td>
<td>0.43</td>
</tr>
<tr>
<td>Wavelength (nm)</td>
<td>289</td>
<td>100.64</td>
<td>0.53</td>
</tr>
<tr>
<td></td>
<td>294</td>
<td>100.64</td>
<td>0.53</td>
</tr>
</tbody>
</table>

*Mean (n = 6); RSD: Relative Standard Deviation.
The conditions under which the drug stability test in solution should reproduce the actual handling conditions of specimens still had to be performed for at least the dissolution time of the whole experiment. As it can be seen in Table 5, all samples remained within the acceptable range established of 98 to 102% during the analysis period. The ANOVA demonstrated that there are no significant differences between the analyses carried out in different periods and calculated $F (F_{cal} = 0.699)$ and tabulated $F (F_t = 4.256)$ for $p < 0.05$.

Table 5. Data for sample stability.

<table>
<thead>
<tr>
<th>Time (hour)</th>
<th>Concentration* SS (%)</th>
<th>Concentration* DS (%)</th>
<th>Concentration* DsS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100.00</td>
<td>99.49</td>
<td>99.80</td>
</tr>
<tr>
<td>12</td>
<td>98.78</td>
<td>99.13</td>
<td>99.25</td>
</tr>
<tr>
<td>24</td>
<td>100.79</td>
<td>100.17</td>
<td>100.19</td>
</tr>
<tr>
<td>36</td>
<td>102.64</td>
<td>100.23</td>
<td>100.31</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>1.61</td>
<td>0.54</td>
<td>0.48</td>
</tr>
</tbody>
</table>

*Mean (n = 3), SS = standard sample, DS = dosage sample, DsS = dissolution sample.

Conclusion

The analytical method proposed to quantify Prop in the biphasic system by UV spectrophotometric technique, was linear, precise, accurate and robust with respect to changes in wavelength, solvent mark and sonication in a concentration range of 0.8 to 96.0 μg mL⁻¹. In addition, the method remained stable in the analyzed experimental conditions, showing to be a method capable of generating highly reliable results and therefore able to be used in the laboratory quality control routine.

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