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Formulation development of immediate release chlorpropamamide tablets using directly compressible excipients

Shahzad Ahmad², Muhammad Harris Shoaib², Rabia Ismail Yousuf², Wajiha Iffat¹*, Sadia Shakeel¹, Najia Rahim¹, Riffat Yasmin¹, Rehana Bibi², Sadaf Ibrahim² and Faisal Muhammad Khan¹

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Diabetes mellitus is a major cause of morbidity and mortality worldwide. Chlorpropamide is an oral antidiabetic drug belonging to sulphonyl urea group, frequently prescribed in the treatment of type II diabetes mellitus. The present study was aimed on adopting cost effective direct compression method for the development of immediate release tablets of chlorpropamide by employing Avicel PH 102 and pregelatinized starch as directly compressible excipients. Each batch of tablets was evaluated for different pharmacopeial and non-pharmacopeial tests. All pharmacopeial tests were within the specified limits. Disintegration time was less than 15 min, except for one formulation. Dissolution results were satisfactory and within the range of 73 to 91% for all formulations. High performance liquid chromatography (HPLC) method was used to determine the drug content of each tablet batch. Assay was within the United States Pharmacopeia (USP) limit for three formulations. The present study concluded that chlorpropamide tablets can be successfully prepared by direct compression method. This technique reduces manufacturing cost by employing least number of excipients. Formulation F3 proves to be the best immediate release formulation because of its good physical attributes and assay results predicting to have improved bioavailability.

Key Words: Chlorpropamide, immediate release tablet, directly compressible excipients, Avicel PH 102.

INTRODUCTION

Chlorpropamide (CP) is an oral glucose lowering agent belonging to sulphonyl urea group effectively used in the treatment of diabetes mellitus.² It increases the secretion of insulin from pancreas by interacting with...
Table 1. Formulations ingredients of chlorpropamide tablets.

<table>
<thead>
<tr>
<th>Formulation ingredient</th>
<th>Amount per tablet (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorpropamide</td>
<td>250</td>
</tr>
<tr>
<td>Avicel PH 102</td>
<td>90-100</td>
</tr>
<tr>
<td>Pregelatinized starch</td>
<td>82-101</td>
</tr>
<tr>
<td>Magnesium stearate</td>
<td>4.5</td>
</tr>
<tr>
<td>Talc</td>
<td>4.5</td>
</tr>
</tbody>
</table>

Adenosine triphosphate (ATP) sensitive potassium-channel receptors on the pancreatic cell surface. It also enhances the effect of insulin on liver. It has a molecular weight of 276.74 Da (Huupponen and Lammintausta, 1981). Figure 1 illustrate the structure of Chlorpropamide. Recommended daily dose ranges from 250 to 500 mg. CP reaches the highest circulating levels in 2 to 4 h, as it is promptly absorbed in the gastrointestinal tract. CP is metabolized in the liver (80%), mainly via CYP2C9 and the metabolites are chiefly excreted in the urine (Arrigoni et al., 1987). Its biological half-life is 36 h. Due to the protracted elimination of the drug; the dose should be reduced by 50% in patients with renal impairment (Abe et al., 2011).

Direct compression offers a number of advantages and considered to be the method of choice for the formulation of tablets over other manufacturing processes. Some distinguishing features of this method include cost effectiveness, suitability for moisture and thermostable active pharmaceutical ingredients (API), faster disintegration and dissolution, etc. Disintegration or dissolution is the rate limiting step in absorption of poorly soluble drugs prepared by wet granulation. The tablets prepared by direct compression reveal relatively more rapid dissolution because the tablets disintegrate into drug particles that directly approaches the dissolution fluid (Jivraj et al., 2000; Rubinstein, 2000). The execution of direct compression formulation generally depends upon the use of appropriate excipients. Excipients compatibility with APIs, particle size distribution, binding capability and flow properties of powder blend are major designing features for directly compressible formulations (Bolhuis and Chowhan, 1996; Parrott, 1990).

These features are offered by a small number of directly compressible binders-fillers (Wade and Weller, 1994). Previous studies had shown that Avicel PH 102 showed excellent compact hardness because of its plastic deformation under pressure (Bolhuis and Chowhan, 1996; Lee et al., 2000). Avicel PH 102 showed better flow than Avicel PH 101, due to its larger particle size and the spherical particle shape (Bolhuis and Chowhan, 1996; Lee et al., 2000). Pregelatinized starch is a modified starch used as a binder, disintegrant and diluent in oral dosage forms. It shows enhanced flow and compression characteristics as compared to simple starch and used in dry-compression or direct compression processes (Iskandarani et al., 2001).

Diabetes mellitus is a major cause of morbidity and mortality globally. Literature shows that the incidence of type II diabetes has more than doubled in the last 30 years (Fox et al., 2006). Among other antidiabetic agents CP is a safe and low cost drug frequently prescribed for the treatment of type II diabetes mellitus. The present study was aimed to develop immediate release CP tablet by cost effective direct compression method.

MATERIALS AND METHODS

Formulations ingredients and reagents

Chlorpropamide (Zafa Laboratories Pvt. Ltd.), avicel PH-102 (FMC Corporation, USA), magnesium stearate (Merck, Darmstadt, Germany), pregelatinized starch (FMC Corporation), talc (FMC Corporation), hydrochloric acid (Merck, KgaA, Darmstadt, Germany), methanol HPLC grade (RDH, Sigma-Aldrich GmbH, Seelze, Germany), glacial acetic acid (Merck, Darmstadt, Germany), acetonitrile HPLC grade (RDH, Sigma-Aldrich GmbH, Seelze, Germany), and distilled water (Department of Pharmaceutics, Faculty of Pharmacy, University of Karachi, Karachi, Pakistan).

Equipment and instruments

Single punch machine (Erweka, Korsch, Germany), electronic balance (Mettler Toledo B204-S, Switzerland), digital hardness tester (OSK, Fujiwara, Ogawa Seiki Co. Ltd., Tokyo, Japan), friability tester (H Jurgen US Co-GmbH, D2800, Germany), basket rack assembly (Erweka ZT-2, Heusesnstamm, Germany), UV double beam spectrophotometer (Shimadzu Corporation, Kyoto, Japan) and HPLC (LC-10A, SPD-2A) (Shimadzu Corporation, Kyoto, Japan).

Process of chlorpropamide tablets manufacturing by direct compression method

Tablets were manufactured by direct compression method employing single punch machine. Formulations were designed with varying percentages of excipients keeping the drug content constant (Table 1). The varying quantities of Avicel PH 102 and pregelatinized starch are summarized in Table 2. All the ingredients were passed through 20 mesh sieve separately, weighed accurately and blended in a polyethylene bag for 5 min excluding magnesium stearate. Magnesium stearate was added lastly and all ingredients were again mixed for further 5 min. The final blends were compressed directly using round shaped biconcave punches. Figure 1

Evaluation of tablet properties

Each batch of tablets was evaluated for different pharmacopeial and non-pharmacopeial tests such as weight variation, hardness, thickness variation, friability, disintegration, dissolution and assay.
Weight variation is a direct indicator of the dosage form uniformity. Therefore, weight of the tablet has to be routinely measured to ensure the proper amount of the drug in each tablet. Tablets (n = 20) were weighed individually by using electronic balance. Hardness of randomly selected tablets (n = 20) was determined by using digital hardness tester. The results are mentioned as mean ± SD. The thicknesses of twenty randomly selected tablets were determined by using vernier caliper and the data was analyzed to calculate mean ± SD. Tablet thickness also becomes important characteristics in counting tablet using filling equipment. Fifteen tablets of each formulation were weighed and subjected to friability studies. Percentage friability of the tablets was calculated by the following formula:

Percentage friability = \( W_1 - W_2 / W_1 \times 100 \)

Where, \( W_1 \) = weight of tablets before testing and \( W_2 \) = weight of tablets after testing. Disintegration test was performed using basket rack assembly in 900 ml of distilled water maintained at 37 ± 2°C and the disintegration time was noted compared with the disintegration time which was noted. The dissolution test (n = 6) were carried out using USP dissolution apparatus II (paddle method) at 37 ± 0.5°C and 50 rpm in 0.1 N HCl acid. The drug concentration was measured using a UV double beam spectrophotometer at 230 nm (Pharmacopeia, 2005).

**Pharmaceutical assay**

Assay was performed on HPLC according to USP 28 (Pharmacopeia, 2005). The chromatographic procedure was carried out by using a stainless steel column (25 cm × 4.6 mm). The recommended mobile phase is a filtered and degassed mixture of equal volumes of acetonitrile and dilute glacial acetic acid. The detection was carried at a wave-length of 240 nm. The peak area of sample was compared with that of reference standard prepared having working strength of 0.005% using mobile phase. Potency of active drug was calculated by the formula:

\[
\% \text{ Assay} = \frac{\text{Average Peak Area of Sample} \times \text{Conc. of Standard}}{\text{Average Peak Area of Standard} \times \text{Conc. of Sample}} \times 100
\]

**RESULT AND DISCUSSION**

Immediate release tablets of chlorpropamide (CP) were prepared by direct compression employing directly compressible excipients. As the powder blend has good flow properties (Table 3). The tablets prepared showed less weight variation. Average tablet weight was in the range of 450.4 to 452.8 mg. Avicel PH 102 offered better binding capabilities with improved flow properties. Magnesium stearate was also added as lubricant to impart better flow properties of powder blend in reducing the chances of weight variation. Thickness variation of CP tablets was in the specified limit of ± 5%. Variation in average tablet weight and thickness is often governed by the flowability of the tablet content and filling of the dye (Davies and Newton, 1996). The hardness of the tablets should be within the acceptable limit. Hardness is an in process control test performed during manufacturing, depicting the compaction behavior of the material (Davies and Newton, 1996). Hardness is an in process control test performed during manufacturing, depicting the compaction behavior of the material (Davies and Newton, 1996). The hardness of the tablets should be within the acceptable limit. Too hard tablet may delay the disintegration and release of drug, however soft tablets may not bear shocks, during handling, storage and distribution (Davies and Newton, 1996; Huynh-Ba, 2009). Proper

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>Angle of repose (°)</th>
<th>Carr’s Compressibility index (%)</th>
<th>Hausner ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>21.5</td>
<td>17.3</td>
<td>1.211</td>
</tr>
<tr>
<td>F2</td>
<td>16.9</td>
<td>14.27</td>
<td>1.191</td>
</tr>
<tr>
<td>F3</td>
<td>18.35</td>
<td>15.13</td>
<td>1.173</td>
</tr>
<tr>
<td>F4</td>
<td>19.93</td>
<td>15.76</td>
<td>1.184</td>
</tr>
</tbody>
</table>
Table 4. Physical testing of chlorpropamide tablets.

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>Weight variation (mg) Mean±SD</th>
<th>Thickness variation (mm) Mean±SD</th>
<th>Hardness variation (kg) Mean±SD</th>
<th>Friability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>451.70 ± 3.32</td>
<td>3.15 ± 0.03</td>
<td>8.61± 0.50</td>
<td>0.663</td>
</tr>
<tr>
<td>F2</td>
<td>451.45 ± 2.52</td>
<td>3.15 ± 0.03</td>
<td>8.47 ± 0.75</td>
<td>0.671</td>
</tr>
<tr>
<td>F3</td>
<td>450.40 ± 3.20</td>
<td>3.15 ± 0.03</td>
<td>8.56 ± 0.73</td>
<td>0.576</td>
</tr>
<tr>
<td>F4</td>
<td>452.8 ± 3.12</td>
<td>3.15 ± 0.03</td>
<td>8.87 ± 0.64</td>
<td>0.619</td>
</tr>
</tbody>
</table>

Table 5. Results of pharmacopeial test of chlorpropamide tablets.

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>Disintegration time (min)</th>
<th>Dissolution (%)</th>
<th>Assay (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>25</td>
<td>80</td>
<td>95.8</td>
</tr>
<tr>
<td>F2</td>
<td>15</td>
<td>73</td>
<td>88.16</td>
</tr>
<tr>
<td>F3</td>
<td>8</td>
<td>91</td>
<td>99.03</td>
</tr>
<tr>
<td>F4</td>
<td>12</td>
<td>79</td>
<td>92.15</td>
</tr>
</tbody>
</table>

Figure 1. Structural formula of chlorpropamide.

selection of the filler/binder and the applied compressional force are decisive factor for tablet hardness. A slight variation in the binder ratio in especially in directly compressed tablet can lead to common defects including chipping, lamination, capping and friable tablets (Peck et al., 1989). Avicel PH 102 was used as binder and filler to overcome these problems. The developed CP tablets have the hardness in the range of 8.47 to 8.87 kg. The friability was also determined and found to be less than 0.7%, demonstrating good mechanical strength (recommended limit is < 1%) (Table 4). Tablet should be assessed for friability to ensure that they are able to sustain their physical integrity when subjected to mechanical shocks during manufacturing and transportation (Huynh-Ba, 2009).

Oral tablet should be disintegrated within the specified limits to release its drug content. The choice of suitable excipients should be carefully executed to get desired disintegration time (Vadas et al., 1984). Most of the developed CP tablets were disintegrated within 15 min except formulation F1 which disintegrated in 25 min (Table 4). Pregelatinized starch was used in the formulation as disintegrant and improves the disintegration of the CP tablet. Another researcher also observed the reduction in the disintegration time of tablets with increase in quantity of starch (Mohammed, 2013).

Dissolution tests were performed using USP dissolution apparatus II in recommended medium that is, acetyl buffer and purified water. For proper absorption, drug release from dosage forms has to be carefully observed. The percentage drug dissolved in 30 min was in the range of 73 to 91%. The drug contents was uniform across all the developed batches of CP tablets and found within the pharmacopeial limits (that is, 90 to 110%) USP 28 (Table 5). The chemical assay was performed using HPLC as mentioned in USP official monograph of CP. HPLC gave accurate and reliable results (Fong, 1991). The pharmacotechnical attributes of developed CP tablets were compared and it was found that F3 is the optimal formulation, with short disintegration time, better
dissolution and drug content near to 100%. Development of oral tablets involves choice of appropriate technique and excipients. In case of immediate release tablet, efficient disintegrants fight against the effectiveness of tablet binder and compressional forces and immediately release the drug content from the tablet. The present study concluded that the combination of pregelatinized starch and Avicel PH 102 employing direct compression provided the formulation to release and sustain the integrity of tablet.

Conflicts of Interest

All authors report no conflict of interest.

REFERENCES


In vitro effects of Eugenia pyriformis Cambess., Myrtaceae: Antimicrobial activity and synergistic interactions with Vancomycin and Fluconazole

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The Eugenia pyriformis Cambess. species, Myrtaceae, also known by the popular name as uvaia was evaluated for its antimicrobial activity. Broth microdilution method was used to determine the Minimum Inhibitory Concentration (MIC) against selected pathogenic strains of bacteria, and fungi. Checkerboard method was used to evaluate the synergistic interactions of E. pyriformis with Vancomycin and Fluconazole. The leaf and stem crude extract showed for Gram-positive strains MIC values of 125 and 250 µg/ml and for leveduriform fungi MIC values ranging from 7.81 to 62.5 µg/ml. Ethyl acetate, hydroalcoholic fractions, and leaf acetonic extract showed MIC values between 62.5 and 125 µg/ml for Gram-positive strains. The ethyl acetate fraction and leaf acetonic extract showed MIC values ranging from 7.81 to 62.5 µg/ml for leveduriform fungi; the stem acetonic extract MIC value was 62.5 µg/ml against Gram-positive strains and MIC value of 7.81 µg/ml for leveduriform fungi. The combination of E. pyriformis with Vancomycin and Fluconazole showed synergistic activity for strains of Enterococcus faecalis, Candida albicans, Candida krusei and Candida parapsilosis with fractional inhibition concentration indices (FICI) below of 0.5. The extracts and fractions of this medicinal plant were able to inhibit the growth of bacteria and fungi in vitro.

Key words: Eugenia pyriformis Cambess, antimicrobial activity, synergistic interaction.

INTRODUCTION

Medicinal plants produce a variety of compounds that show biological activities, which are employed for developing drugs, representing a source of great importance in research of new antimicrobial agents (Newman

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These compounds derive from several secondary metabolic pathways, and include alkaloids, flavonoids, lignins, phenolic compounds and terpenoids (Saleem et al., 2009).

The extensive use of antimicrobials has led to growing resistance and the spread of many bacterial and fungal pathogens, which now constitutes a serious medical problem. The combination of antimicrobial therapy has become an alternative for the treatment of infectious diseases caused by multiresistant bacteria (Wolska et al., 2012). Essential oils, extracts and isolated compounds containing secondary metabolites are able to delay or inhibit bacteria, yeasts and levedufurin fungi growth (Tiwari et al., 2009). These compounds display antimicrobial activity when used alone, but there is also the possibility of using them in combination with conventional antimicrobials in order to improve their efficacy (Wolska et al., 2012).

The Myrtaceae family considered the most complex from the taxonomic point of view shows in its leaves a great amount of volatile constituents (Stieven et al., 2009). It is widely found in the Americas and Oceania, and in Brazil, it is represented by 23 genera and a thousand species distributed all over the country, mainly through the Atlantic Forest and restinga, with about a third of these species belonging to the Eugenia genus (Landrum and Kawasaki, 1997; Farias et al., 2009).

The species Eugenia pyriformis Cambess, representative of this family, is a common plant in the states of São Paulo, Paraná, Santa Catarina and Rio Grande do Sul, known by the popular name of uvaia, uvaieira, uvaia-do-campo, uvalha or uvalha-do-campo (Armstrong et al., 2012). The plant is grown in orchards and employed in popular medicine, its blooming occurs from November to January and edible fruits ripen becoming yellow in January and February, and they present high levels of antioxidant activity and phenolic compounds (Stefanello et al., 2009).

The uvaia is a plant that can be used in reforestation programs, showing easy cultivation and growing in gardens, its rich nutritional value fruits are used in industrial manufacturing of several products (Lorenczi et al., 2006) and its leaves act in treatment for gout (Schmeda-Hirschmann et al., 1987; Theoduloz et al., 1988). The fruit extract of E. pyriformis showed antimicrobial activity against Enterococcus faecalis, Staphylococcus aureus and Pseudomonas aeruginosa (Stieven et al., 2009).

Given the importance of Myrtaceae family and due to scarce studies conducted so far, this plant represents a great potential of exploration and a promising field for development of antibacterial and antifungal agents for treatment of human and animal infections. This study aims at evaluating the in vitro antimicrobial activity and potential synergistic of the extracts and fractions of E. pyriformis Cambess.

MATERIALS AND METHODS

Plant and preparation

Aerial parts of E. pyriformis Cambess were collected in campo limpo and in bords de capão at Curitiba’s Jardim Botânico, under the coordinates 25° 26' S; 49° 14' W, at an altitude of 330 m, in June. The plant identification was performed by the botanist Gert Hattchbach, at Botanical Garden of Curitiba (MBM) herbariums, under number 204990.

The crude ethanolic extract was prepared with 96° GL ethanol, in continuous reflux for 6 h, at 50°C in modified Soxhlet device. Fractions were obtained through the liquid-liquid partitioning method. In the technique, solvents of analytical standard PA were used in increasing order of polarity (hexane, chloroform and ethyl acetate), being the fraction remaining to the hydroalcoholic. The crude aceton extract was obtained from leaves and stem extracted with acetone at 30°C during a period of 6 h in modified Soxhlet device.

The screening phytochemical was performed in thin layer chromatography (TLC) silica gel 60 F254 (Merck) analysis of the crude ethanolic extract, fractions and crude aceton extract in mobile phase and reveals specific to indicate the presence of steroids/triterpenoids (vanillin-sulfuric acid 1%), tannins (ferric chloride 1%) and phenolic compounds (Neu-reagent). The following solvents moistures were used toluene/ethyl acetate (97:3) for steroids/triterpenes, ethyl acetate/formic acid/glacial acetic acid/water (100:11:11:27) for tannins and phenolic compounds.

For the microbiological analysis, the extracts and fractions were prepared in 10% ethanol and 2% dimethyl sulfoxide (DMSO), and filtered through 0.22 μm Millipore membrane (TPP, Trasadingen, Switzerland) in order to assure its sterility.

Antibacterial activity

The antibacterial activity tests were performed with the following strains: Enterococcus faecalis ATCC 29212, S. aureus ATCC 25923, Escherichia coli ATCC 25922, Klebsiella pneumoniae ATCC 700603 and P. aeruginosa ATCC 27853.

The Minimum Inhibitory Concentration (MIC) values were determined through broth microdilution method (Clinical and Laboratory Standards Institute [CLSI], 2008a). Bacterial suspensions were prepared in saline solution at concentration of 1.0 × 10^8 CFU/ml, corresponding to 0.5 McFarland tube and they were subsequently inoculated in a 5 µl volume into the wells, thus remaining a final concentration of 10^6 CFU/ml.

The inhibitory activity negative control of the diluents, ethanol and DMSO was prepared by adding 100 µl of 10% ethanol and 2% DMSO solution in 100 µl of Mueller-Hinton broth (MBH) and 5 µl of the bacterial inocula. For the sterility control, 100 µl of MBH and 100 µl of the extract or fraction were used. The bacterial viability or positive control was prepared with 100 µl of MBH and 5 µl of the bacterial inocula.

Microplates were incubated in bacteriological incubator at 35°C for 16 to 20 h. After this time interval, 20 µl of aqueous solution of 0.5% Triphenyltetrazolium Chloride (TTC – Merck, Darmstadt, Germany) were added, and the microplates were incubated again for 3 h at 35°C. The results reading were subsequently performed, where the red coloration formation in the wells was interpreted as absence of antimicrobial activity for the studied substance.

For the results analysis, the MIC values obtained were classified as having good inhibitory potential (up to 100 µg/ml); moderate inhibitory activity (between 100 and 500 µg/ml); weak inhibitory activity (between 500 and 1000 µg/ml), and absence of inhibitory activity (higher than 1000 µg/ml) (Ayres et al., 2008).
Table 1. Antimicrobial activity of stem and leaves of *E. pyriformis* Cambess.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Stem extracts and fractions (µg/ml)</th>
<th>Leaf extracts and fractions (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RE</td>
<td>HEF</td>
</tr>
<tr>
<td><strong>Gram-positive</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. faecalis</em> ATCC 29212</td>
<td>250</td>
<td>500</td>
</tr>
<tr>
<td><em>S. aureus</em> ATCC 25923</td>
<td>250</td>
<td>-</td>
</tr>
<tr>
<td><strong>Gram-negative</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> ATCC 25922</td>
<td>1000</td>
<td>-</td>
</tr>
<tr>
<td><em>K. pneumoniae</em> ATCC 700603</td>
<td>1000</td>
<td>-</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> ATCC 27853</td>
<td>1000</td>
<td>-</td>
</tr>
<tr>
<td><strong>Levedufurform fungi</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. albicans</em> ATCC 40175</td>
<td>31.25</td>
<td>-</td>
</tr>
<tr>
<td><em>C. krusei</em> ATCC 40174</td>
<td>31.25</td>
<td>-</td>
</tr>
<tr>
<td><em>C. parapsilosis</em> ATCC 40038</td>
<td>62.5</td>
<td>-</td>
</tr>
</tbody>
</table>


**Antifungal activity**

The tests were performed with the *Candida albicans* ATCC 40175, *Candida krusei* ATCC 40147 and *Candida parapsilosis* ATCC 40038 strains.

Serial dilutions of the extracts and fractions in a concentration range from 1000 to 7.81 µg/ml were prepared with liquid medium RPMI 1640 (Gibco/Invitrogen, New York, USA) in 96-well, U-shaped bottom sterile microplates (CLSI, 2008b). The distinct fungal suspensions were prepared in saline solution at initial concentration of 1.0 × 10^6 CFU/ml. These suspensions were diluted in liquid medium until a 1.0 to 5.0 × 10^9 CFU/ml final concentration was reached and subsequently inoculated 100 µl into the wells. The microplates were incubated for 48 h at 35°C. After this period, 20 µl of 0.5% TTC were added and the plates were incubated again for 3 h at 35°C. The results reading and analysis were performed according to the same methodology as the antibacterial activity.

**Synergistic activity**

The analyses of synergism were determined through Checkerboard method using extracts and fractions of *E. pyriformis* that showed MIC values below 100 µg/ml in combination with the antimicrobials Vancomycin and Fluconazole.

The antimicrobial in the combination was serially diluted along the ordinate of the microplate, while the extracts and fractions were diluted along the abscissa. The concentrations were prepared corresponding to MIC/8, MIC/4, MIC/2, MIC, MICx2 and MICx4. The combination for each reference strain was tested in duplicate. The first antagonistic, additive or synergistic effect of the extracts and fractions in combination with the antimicrobial was determined with calculation of fractional inhibitory concentration indices (FICI). FICI was calculated as FICA + FICB, where FICA = MICA of the combination/MIC of the combination alone. The results were interpreted as synergism (FICI < 0.5), addition (0.5 < FICI > 4) or antagonism (FICI > 4) (Chung et al., 2011).

The second method involved plotting the data as isobolograms (Hemaiswarya and Doble, 2010). The graph is represented with the ratio to the FIC of the *E. pyriformis* on the x-axis and the ratio of the FIC of the antimicrobial on the y-axis. A straight line that connects the ratio 0.5 in the ordinate 0.5 in the abscissa indicates the line synergism. A straight line that connects the ratio 4.0 in the ordinate 4.0 in the abscissa indicates the line additivity, the location of the FIC of the combination considerably above the line indicates antagonism.

**RESULTS AND DISCUSSION**

The antimicrobial activity in vitro of extracts and fractions of stem and leaves of *E. pyriformis* Cambess was determined in this study. The values obtained in the microbiological assays are presented in Table 1.

According to this established profile, the leaf hydroalcoholic and ethyl acetate fractions showed pronounced inhibitory activity for *E. faecalis* and *S. aureus* (MIC=62.5 µg/ml), and the results were considered good in the scale established. Similar results were obtained to stem aceton extract (*E. faecalis* and *S. aureus*) and leaf aceton extract (*E. faecalis*) showed good inhibitory potential (MIC = 62.5 µg/ml).

The phytochemical screening showed the presence of sterols/triterpenes in stem and leaf aceton extract, hexane and chloroform fraction; tannins and phenolic compounds in the aceton extract, chloroform, ethyl acetate and hydroalcoholic fractions. The reagent Neu showed yellow bands which are characteristic of flavonoid compounds (Rifault et al., 2014). Chavas et al. (2014) reported that leaf extracts of *E. pyriformis* showed the presence of alkaloid, flavonoid, tannin, saponin and stem extracts showed tannin and saponin in their composition.

The observed antibacterial activity is attributed to the presence of different bioactive compounds which have an impact on growth and metabolism of microorganisms. The phenols and flavonoids significantly contribute to the
Table 2. FIC indices of E. pyriformis Cambess with Vancomycin and Fluconazole against strains of Gram-positive and leveduriform fungi.

| Compound | E. faecalis ATCC 29212 | | | S. aureus ATCC 25923 | | |
|----------|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|
|          |                         | Gram-positive            |                         |                         |                         |
|          |                         | **E. faecalis ATCC 29212** |                         | **S. aureus ATCC 25923** |                         |
| A        | B                       | *MIC<sub>A</sub> | *MIC<sub>B</sub> | **FIC<sub>A</sub>** | **FIC<sub>B</sub>** | $\delta FIC$ | *MIC<sub>A</sub> | *MIC<sub>B</sub> | **FIC<sub>A</sub>** | **FIC<sub>B</sub>** | $\delta FIC$ |
| AES      | VAN                     | 62.5 | 1 | &gt;8 | &gt;8 | &gt;8 | 62.5 | 2 | 0.12 | 2.00 | 2.12 |
| AEL      | VAN                     | 62.5 | 1 | 8 | 0.12 | &gt;8 | 62.5 | 2 | 1.00 | 0.25 | 1.25 |
| EAFL     | VAN                     | 62.5 | 1 | 8 | 0.12 | &gt;8 | 62.5 | 2 | 0.12 | 2.00 | 2.12 |
| HFL      | VAN                     | 62.5 | 1 | 0.25 | 0.12 | 0.37 | 62.5 | 2 | 0.12 | 2.00 | 2.12 |

<table>
<thead>
<tr>
<th>Compound</th>
<th>C. albicans ATCC 40175</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Leveduriform fungi</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>C. albicans ATCC 40175</strong></td>
<td></td>
<td><strong>C. krusei ATCC 40174</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>B</td>
<td>*MIC&lt;sub&gt;A&lt;/sub&gt;</td>
<td>*MIC&lt;sub&gt;B&lt;/sub&gt;</td>
<td><strong>FIC&lt;sub&gt;A&lt;/sub&gt;</strong></td>
</tr>
<tr>
<td>RES</td>
<td>FLU</td>
<td>31.25</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>REL</td>
<td>FLU</td>
<td>31.25</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>AES</td>
<td>FLU</td>
<td>7.81</td>
<td>0.50</td>
<td>1.00</td>
</tr>
<tr>
<td>AEL</td>
<td>FLU</td>
<td>7.81</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>EAFL</td>
<td>FLU</td>
<td>62.5</td>
<td>0.50</td>
<td>0.25</td>
</tr>
</tbody>
</table>


to the antibacterial activity, can form complexes with cell wall and also disrupt bacterial envelopes (Kurek et al., 2011).

The stem and leaf acetonic extract, hydroalcoholic fractions and leaves ethyl acetate showed good inhibitory potential against Gram-positive microorganisms; however, MIC values above of 250 µg/ml were shown for Gram-negative. Low inhibitory activity presented in Gram-negative bacteria in relation to Gram-positive bacteria could be ascribed to their differences in cell membrane constituents and their arrangement. The resistance of Gram-negative bacteria towards antibacterial substances may be due the presence of outer membrane as a permeability barrier, difficult for compounds diffusion through its lipopolysaccharide membrane (Chew et al., 2011). The absence of this barrier in Gram-positive bacteria allows direct contact of substances with the cell membrane phospholipid layer, thus allowing the increase in ionic permeability and leakage of vital intracellular constituents, or even resulting in its enzymatic systems deficiency (Zarai et al., 2011).

The antifungal activity analysis (Table 1) showed good inhibitory potential for crude extracts, with MIC values ranging from 7.81 and 62.5 µg/ml. These values were the lowest MICs found in relation to all the tested microorganisms. Results suggest an antifungal activity efficiency of E. pyriformis on leveduriform fungi, which despite being eukaryotic organisms, with more complex structural organization in comparison with bacteria (Teke et al., 2011), showed more significant MIC values. The leaves and stem acetonic extract also showed good inhibitory potential on leveduriform fungi (MIC=7.81 µg/ml), and the ethyl acetate fraction showed good inhibitory potential only for the leaf (MIC=31.25-62.5 µg/ml). The other tested fractions did not show any inhibitory activity.

The results of the analysis of synergism of extracts and fractions from E. pyriformis with Vancomycin and Fluconazole were determined against Gram-positive and fungi leveduriform as depicted on Table 2.

The combination of leaf hydroalcoholic fraction and Vancomycin exhibited synergism against E. faecalis with FICI of 0.37 while the combination of Fluconazole with either leaf crude extract or leaf acetonic extract of E. pyriformis showed enhanced
enhanced efficacy against *C. krusei* and *C. parapsilosis* with FICI values ranging between 0.24 and 0.50. On the hand combination of Fluconazole with leaf ethyl acetate fraction showed enhanced efficacy against *C. albicans*, *C. krusei*, and *C. parapsilosis* with FICI values ranging from 0.24 to 0.37.

Representative isobolograms of the combination of extracts and fractions from *E. pyriformis* with Vancomycin and Fluconazole against all the microorganisms are as shown graphically in Figure 1. A synergistic interaction was observed for one combination with Vancomycin and six with Fluconazole for the microorganisms with the FIC below the line of synergism.
Conclusively, extracts and fractions obtained from *E. pyriformis* Cambess showed antimicrobial activity as exhibited by their ability to inhibit bacterial and fungal growth *in vitro*.

**Conflict of Interests**

The author(s) have not declared any conflict of interests.

**REFERENCES**


Full Length Research Paper

Technological development and evaluation on sialagogue activity of a spray-like liquid formulation of pilocarpine

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Xerostomia is a common condition in patients undergoing oncological treatment. As a result of adverse effects of drugs or as an answer to radiotherapy radiation, the salivary glands of mouth stop or reduce the production and secretion of saliva. This leads to severe consequences such as caries, infections, difficulty in swallowing, and sensory loss. Thus, this work aimed to develop a new product to be used in patients suffering from xerostomia and improving salivation added to easy application to promote high acceptance rate. In this way a spray formulation of pilocarpine was developed and evaluated for its in vivo activity (in rats) on salivary stimulation. Pre-formulation, development of spray and quality control studies were performed. The formulation developed was evaluated regarding the ability to improve salivation in adult Wistar rats. There was a significant increase (p<0.05) in salivation produced by spray formulation when compared with oral solution in the same concentrations. The spray formulation is an important tool developed for the treatment and support of patients suffering from xerostomia and optimization of these results should be performed.

Key words: Pharmacology, technology, mouth dryness, pilocarpine, xerostomia, oncology.

INTRODUCTION

Xerostomia is a subjective sensation of dry mouth resulting from a decrease or cessation of salivary glands function with changes in quantity or quality of saliva. Salivary hypofunction is characterized by a quantitative decrease in salivary flow, when it drops to less than 50%, or by a change of saliva composition with loss of mucin, and consequently, reducing lubrication (Coimbra, 2009). It is one of a set of signs and symptoms resulting from certain diseases or various stimuli, represented mainly by irradiation of head and neck, or other cancer treatments, as well as Sjögren’s syndrome, Graft-Versus-Host disease and adverse effects to certain drug therapies. Some systemic diseases can also cause salivary dysfunction, including diabetes, human immunodeficiency virus infection (HIV), Parkinson's disease, Alzheimer's disease and cystic fibrosis (Fávaro et al., 2006; Imanguli...
et al., 2008).

Xerostomia has implications not only physically but also psychologically and socially, with some discomfort for patients due to the feeling of dry mouth and also with the higher number of infections of the oral mucosa and dental caries (Feio and Sapeta, 2005). Xerostomia results in difficulty in swallowing and articulation of words, and a general decline in the ability to eat, talk and sleep (Tolentino et al., 2011). The treatment of these symptoms includes maintenance of hydration, avoidance of tobacco and alcohol, maintenance of good oral hygiene (brushing, chlorhexidine and fluoride to prevent cavities and plaque), and stimulation of the reflex arc, for example, chewing gum with flavors and sugar acids, suited to induce salivation, and also the use of artificial saliva two to three times daily (Coimbra, 2009; Kaluzny et al., 2014).

Finally, the possibility of using cholinergic agents as pilocarpine and cevimeline for stimulating salivation has always been attractive. These agents are cholinergic agonists, acting on M₃ receptors, predominantly expressed in smooth muscle and glandular tissues (Ishii and Kurachi, 2006). This leads to glandular secretion by difference of charges between the spaces in and out of salivary lumen, a process mediated by changes in [Ca²⁺], by the IP₃-mediated Ca²⁺ signalling pathway (Nakamura et al., 2004). When there is still some residual salivary function, saliva stimulants produce greater relief than saliva substitutes or other palliative procedures (Kaluzny et al., 2014).

In the past, pilocarpine has been investigated as a mean of systemic management of xerostomia secondary to irradiation of the head and neck. Currently, systemic pilocarpine is indicated for the management of xerostomia secondary to irradiation damage, chronic Graft- Versus-Host disease and glandular autoimmune attack given by Sjögren’s syndrome (Fávaro et al., 2006; Agha-Hosseini et al., 2007). Furthermore is the sole sialagogue agent approved by FDA for radiotherapy treatment (Kaluzny et al., 2014). Cevimeline, another cholinergic agent, was assessed on its sialagogue activity and compared to pilocarpine. Both drugs showed effect on submandibular and sublingual glands, while cevimeline had stronger side effects in central nervous system (CNS) (Omori et al., 2003). Cevimeline activates common salivary mechanism with pilocarpine, but has a slower onset of activation, longer duration of salivation and an increased pressor response at higher doses. However, the cevimeline has an anti-dipsogenic effect due to the inhibitory neuronal effect on the thirst-related central nuclei (Ono et al., 2012).

Pilocarpine treatment in patients with Sjögren’s syndrome usually starts with 5 mg for a few days, then 5 mg twice daily, for a week, and then, if the patient does not respond, the dose is increased to 15 or 20 mg a day. In some cases the dose can be increased to 30 mg a day (Tsifetaki et al., 2003). Some studies with pilocarpine show that the clinical side effects pointed to a small proportion and is usually characterized by facial flushing, sweating and increased urinary frequency, lacrimation and rhinitis (Nakamura et al., 2009; Bernardi et al., 2002; Kaluzny et al., 2014).

An alternative to avoid these effects would be a local than systemic application of the drug. However, the major difficulty in pilocarpine use does not lie primarily in their side effects, but in adoption of a protocol for these patients and the acquisition of drug. The tablet Salagen® is the only formulation with pilocarpine available on market and it is not sold in Brazil. It demands importation and taxes relative to product, making the treatment very expensive (Neto and Sugaya, 2004).

In Brazil, there are no medications based on salivary stimulation, even with pilocarpine, which has effective action in xerostomia and with significant production in the country, including exportation by VegeFlora Ltd. Company, located in Parnaiba, city from Piauí coast located in Brazil. Thus, the development of national products based on this active principle would result in lower costs and higher compliance, improving quality of life for patients suffering from these symptoms.

The aim of this study was to develop a new presentation for pilocarpine based on a spray formulation. Then to realize a pre-clinical trial using rats, aiming to evaluate the spray efficacy regarding to an oral solution of pilocarpine, to compare the spray formulation with a solution representing the current formulation in the market (the oral tablets labelled as Salagen®). This methodology of sialometry aims to evaluate answers (increase or decrease in salivation), as a simple test that can be used in further studies.

MATERIALS AND METHODS

Development of spray formulation

The development of the formulation initially involved the choice of excipients, determined by the capability to increase the duration of drug action and to improve the viscosity, taste and flavor of the formulation. The pilocarpine hydrochloride (active ingredient) was obtained from VegeFlora Company (Parnaiba city, Piauí, Brasil) and its identification was carried out by the method of Fourier transform infrared spectroscopy (FT-IR Spectroscopy) in IR Spectrum 100 brand PerkinElmer KBr cell apparatus, with the range 4000 to 450 cm⁻¹. The others excipients (Honey, methylparaben, propylparaben, glycerin, saccharin sodium cyclamate, menthol and Hydroxypropyl cellulose ° Klucel®) used in formulations were purchased in a manipulation pharmacy, located in Teresina, city of Piauí, Brasil. Alcohol was used in very low quantitative, just to facility the preservatives solubilization. Later, the excipients were mixed at room temperature.

The following parameters were evaluated: organoleptic characters, pH (in equipment brand “Hanna,” Model PH21), density (second method described in general methods of the 5th edition of Brazilian Pharmacopoeia, with the help of pycnometer with a capacity of 5 ml), spraying volume (50 sprinklings in the bottle valve, driven in a graduated cylinder to measure the corresponding volume) and assay (high resolution chromatography (HPLC), with column LiChroCARTSuperspher 125-4 100 RP-18 end capped, and
Sialometry: Initial weight of cotton; Cotton inserted into the oral cavity; Last weight, after 7 min;

Spray: 3 spray (0.3 ml); Time until sialometry: 10 min; 2, 4 and 14 µmol/kg

Oral: 0.3 ml of pilocarpine solution; Time until sialometry: 30 min; 2, 4 and 14 µmol/kg

Standard: Cleaning of mouth with cotton; Administration of vehicles for both formulations; Time until sialometry: 10 min for spray and 30 min for oral solution.

Figure 1. Sialometry study represented in a chronological flowchart of actions.

Sialometry study using Wistar rats

This study (protocol n. 038/09) is in agreement with the Ethical Principles in Animal Experimentation, adopted by the Ethics Committee on Animal Experimentation at Piauí Federal University (CEEA/UFPI) and was approved in 2009.

Pre-clinical trials were performed using male Wistar rats from vivarium of Agricultural Sciences Center (UFPI), three months of age and weighing between 270 and 290 g. This trial involved the sialometry methodology described by Takakura et al., (2009), with concentrations of 2, 4 and 14 µmol/kg of pilocarpine inserted into a spray formulation (test) and in an oral formulation (standard, solution of pure water and pilocarpine hydrochloride). The concentrations were chosen based on the oral formulation of Salagen® adapted for the animal’s weight, in experiment.

Fifty six rats were divided in eight groups. Two of them received only vehicles of oral (water) and spray formulation. In the other six groups the standard and test formulation were administered in the concentrations of 2, 4 and 14 µmol/kg of pilocarpine. This sialometry study was presented in a chronological flow chart of actions. Rats were anesthetized with sodium pentobarbital (40 mg/kg) via the intraperitoneal route (i.p.). The oral formulation administered using a syringe linked to a cannula, which was inserted in the throat through the mouth to ensure the swallowing of the whole oral solution. The administration of the test formulation was realized using a tweezer to slightly open the mouth so that the spray could be applied. After 10 min weighed cotton ball was introduced to the oral cavity, with a tweezer’s help, while the rat was in the lateral decubitus. The cotton balls were removed after 7 min and weighed again to measure the saliva production (Figure 1).

RESULTS

The active principle was evaluated by infrared as shown in Figure 2, being obtained as a spectrum with intense absorption bands between 2800 and 3200 cm⁻¹, 1600 and 1800 cm⁻¹ and a last one between 1100 and 1035 cm⁻¹. The pilocarpine formulation was developed as shown in Table 1. The formulations evolved until pilot 3, which shows best results regarding to quality control tests and organoleptic characterises and therapeutic necessities by future patients.

With the formulation chosen, tests regarding to quality control were realized to determine its characteristic to assure that it was utilized as a specific and adequate formulation in preclinical test. Then the sialagogue activity test was performed and plotted as shown in Figure 3. In this picture four groups were plotted for three different concentrations of pilocarpine (2, 4 and 14 µmol/kg). These groups include the placebo for both formulations (oral solution and spray), and the two formulations with their respective concentrations. The figure shows the
Figure 2. Identification of pilocarpine hydrochloride by the method of infrared spectroscopy, carried out in IR Spectrum 100 brand PerkinElmer KBr cell, in the range of 4000 to 450 cm\(^{-1}\).

Table 1. Composition of the liquid formulation designed to spray formulation of xerostomia.

<table>
<thead>
<tr>
<th>Composition</th>
<th>Function</th>
<th>Formulation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pilocarpine P.A.</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Honey</td>
<td>Thickener, Sweetener</td>
<td>30</td>
</tr>
<tr>
<td>Methylparaben</td>
<td>Preservative</td>
<td>0.1</td>
</tr>
<tr>
<td>Propylparaben</td>
<td>Preservative</td>
<td>0.02</td>
</tr>
<tr>
<td>Alcohol</td>
<td>Solubilizer</td>
<td>sq</td>
</tr>
<tr>
<td>Glycerin</td>
<td>Sweetener / Thickener</td>
<td>6</td>
</tr>
<tr>
<td>Saccharin</td>
<td>Sweetener</td>
<td>0.06</td>
</tr>
<tr>
<td>Sodium cyclamate</td>
<td>Sweetener</td>
<td>0.05</td>
</tr>
<tr>
<td>Menthol</td>
<td>Flavor/Thickener</td>
<td>0.06</td>
</tr>
<tr>
<td>Sodium hydroxide</td>
<td>Alkalizing</td>
<td>sqf</td>
</tr>
<tr>
<td>Hydroxypropylcellulose</td>
<td>Mucoadhesive</td>
<td>0.3</td>
</tr>
<tr>
<td>Purified water sqf</td>
<td>Vehicle</td>
<td>100</td>
</tr>
</tbody>
</table>

X: Quantity determined for the in vivo study, sq: sufficient quantity, sqf: sufficient quantity for.

DISCUSSION

In infrared spectrum, the intense absorption band between 2800 and 3200 cm\(^{-1}\) suggests similarities with the amine attached to aromatic carbon representing the substituted imidazole ring. Another absorption band was observed in 1770 cm\(^{-1}\) which means typical double bond between carbon and oxygen (C = O) in lactone rings, and in 1620 cm\(^{-1}\), C = C bond of the aromatic type, both indicating the presence of the second ring constituent of the pilocarpine hydrochloride molecule (Silveira, 2010). The pilocarpine analysed was in its hydrochloride salt presentation, which was evidenced by the presence of the peak between 1100 and 1035 cm\(^{-1}\), representing the connection between carbon and chloride in aromatic ring (Pavia et al., 1996).

After confirming the presence of active principle, the study continued with development of a spray formulation as shown in Table 1. The formulation should contain ingredients to promote safety and adhesion to treatment. Thus it has the presence of preservatives, sweeteners and vehicle, resulting in an aqueous formulation, colourless and with sweet taste. Saccharin is 300 to 600 times sweeter than sucrose and frequently used in tablets, oral care products and oral gain and stop of gain in salivation for both formulations. Added with physiological behaviour observations, these results allow to compare which has more efficacy and safety. The results were analysed statistically by ANOVA and t-Student-Newman-Keuls as post hoc test.
pharmaceutical formulations, in concentrations from 0.02 to 0.5% w/w (Rowe et al., 2009). However, in approximately 25% of the population saccharin leads the feeling of metallic or bitter taste, even in normal doses, which can be masked by the addition of other sweeteners agents in low concentrations. Therefore, a second sweetener was included, glyc erine, which is clear, odourless and approximately 0.6 times sweeter than sucrose. Besides, glyc erine is a sticky and hygroscopic agent, giving also a higher viscosity to the formulation (Rowe et al., 2009).

Beside this was introduced honey and sodium cyclamate, two sweeteners. The choice of honey was influenced by his agreeable aroma and flavor, and also medicinal properties have long been known. When applied to the oral mucosa of patients undergoing radiotherapy for example it appears to offer an additional benefit, limiting the severity of mucositis, often presented by these patients. In another study, patients with head and neck cancer were treated with honey, exclusively, with a significant reduction in the discomforting symptoms of mucositis (Bardy et al., 2008).

Therefore, honey was added for its healing properties, stimulating tissue growth, anti-inflammatory and antibacterial properties, reducing the discomfort and the emergence of infections (caries, gingivitis, etc.), especially in irradiated patients (Khanal et al., 2010).

The addition of hydroxypropyl cellulose, a bioadhesive polymer, was used to work like a matrix for controlled release of drugs. Formulations designed for delivery to the mouth have the problem of high swelling, which leads to a low contact time between drug and surface. Thus, its mucoadhesive property drew attention to the benefits of forming a film on the oral mucosa, prolonging the local effects of the drug on the salivary glands (Rowe et al., 2009).

As the aroma directly affects the reflex response to increased salivation, to make it even more pleasant and attractive, was introduced menthol. It is a flavouring agent that gave a pleasant aroma to the formulation, exerting a fresh feeling probably by direct interaction with cold receptors in the body, a fact exploited in most commercial topical presentations, mainly oral (Rowe et al., 2009).

The formulation presented sensory parameters of pleasant smell of menthol and honey, sweetness flavor and cooling sensation, and optimum viscosity to remain longer on the oral mucosa, as compared with liquid formulations, which have low viscosity and tend to be swallowed faster. The pH was 3.45 ± 0.12, below the normal pH of the oral mucosa which was determined by the presence of acidifying agents such as sucrose (pH = 2.0 in 0.35% w/v), hydroxypropylcellulose (pH = 5.0 to 8.5 in 1% w/w), sodium cyclamate (pH=5.5 to 7.5 in 10% w/v) (Rowe et al., 2009). However, it is an oral formulation that should be compatible with physiological pH, avoiding discomfort, irritation or even damage to the mucosa with the drug (Bhanja et al., 2010). The physiological pH of mouth is kept within the range of 6 to 7. However may vary between 5.3 (at low flow rates) and 7.8 (in peak salivation) (Humphrey and Williamson, 2001).

Despite the possibility of use of lemon juice or citric acid 2% on the back of the tongue to stimulate salivation in normal conditions, the xerostomia patients already suffer with the consequences of acidity (Feio and Sapeta, 2005). To avoid this potential problem, is important as the addition of sodium hydroxide until the adequate pH in the

\[ \text{Pilocarpine hydrochloride (µmol/kg)} \]

\[ \begin{array}{cccc}
2 & 4 & 14 & \\
\end{array} \]

\[ \begin{array}{cccc}
\text{Sialogogue activity production of saliva (mg)} & \text{Vehicle - Oral Solution} & \text{Oral Solution} & \text{Vehicle – Spray} & \text{Spray} \\
\end{array} \]

Figure 3. Sialometry analyse in Wistar rats of pilocarpine spray and pilocarpine in oral solution. aP < 0.05 when compared to the lowest concentration; bP < 0.05 when compared to the intermediate concentration; cP < 0.05 when compared to the lowest and higher concentrations, *P < 0.05 when comparing vehicle to any formulation: (ANOVA and t-Student-Newman-Keuls as post hoc test).
range of 6 to 7.

The bulk density obtained was 1.1022 g/ml, above the density of water. The volume for sprinkling, important to determine the dosage of the new medicine, resulted in 0.1 ml/sprinkling. Three spray formulations were produced, named F1, F2 and F3. The theoretical concentrations of the three formulations were equivalent to the doses of 2, 4 and 14 µmol/kg, according to previous studies in xerostomia. Adjusted to the average weight of the adult rats (280 g), and the dose administered to each one determined as three sprinklings (0.3 ml), resulted in formulations with 0.45, 0.91 and 3.2 mg/ml of pilocarpine, respectively.

To confirm this theoretical concentration, an assay study made in high performance liquid chromatography (HPLC), showed that F2 had approximately two times more active than F1 and the F3 had 3.5 times than F2. Thus no interaction occurred between the active and the excipients, making the formulation appropriate for the drug in use and able to be performed in sialometry study. With the spray formulation already developed, the test of sialogogue activity was performed with results as shown in Figure 3. First, the statistical analysis of placebos from oral solution and spray showed a significant increase (p < 0.05) in salivation produced by the second, justified by the advantages given by its excipients, which act as adjuvants in salivary stimulation acting in salivary reflex. These excipients are represented mainly by the honey and menthol. The attractive aroma and the sweet supply (honey) present in the formulation, induce the gustatory memory, with the emergence of primitive reflex responses such as licking the lips and saliva (Guyton and Hall, 2006) which was observed in healthy rats, without damage to the salivary glands.

An oral solution of pilocarpine and water, a systemic presentation, were used to compare with the spray, the local presentation. The advantage of the spray can be observed in the significant increase (p < 0.05) obtained with the concentration of 4 µmol/kg compared to the oral solution. When comparing the group of 14 µmol/kg spray with the lower doses (placebo group, spray 2 and 4 µmol/kg) the salivation increased significantly. However, the variation between responses in doses of 4 and 14 µmol/kg was very small. Higher doses should be avoided when there is no comparable improvement and more side effects are likely to emerge (Santana, 2009).

In the oral solution, a significant increase of salivation was observed with greater dose compared to the other groups of oral solution and placebo. However, this variation was not linear. The oral group of 14 µmol/kg had greater than the acceptable variation within the population sample, with a standard deviation of ±45. The error in this group suggests the exaggerated increase in stimulating effect (excitatory) that the systemic concentration produces in central nervous system (CNS). Among the typical side effects, the animals developed diarrhoea, increased urination, cardiac abnormalities, and muscle contractions in some rats, which have been cited in the literature like toxicity effects of cholinergic agonists (Santana, 2009).

In this way, the spray formulation showed better results in lower doses. Despite its lower effects in 14 µmol/kg dosages, it did not present side effects like the oral solution, which systemically led to intolerable side effects. Thus, when compared with the oral solution and the spray in 4 µmol/kg, a significant increase was observed demonstrating the efficiency and quickly action of oral topical formulation.

**Conclusion**

The spray formulation of pilocarpine hydrochloride, with all the excipients chosen, showed promising results to induce salivation in preclinical studies, with better results in lower doses and even with high doses showed more safety than oral solution with the same active drug. A patent application has been made with the INPI. Clinical trials will be conducted to ensure this application.

**REFERENCES**


