In Vitro Release Study of Nystatin from Chitosan Buccal Gel

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ABSTRACT

Bioadhesive nystatin gel formulations with chitosan as gelling agent were developed and evaluated for in vitro release properties and antifungal activity. Different concentrations of nystatin (1, 2 and 3% w/w), chitosan (5, 6 and 7% w/w) and various solubilizing agents (glycerol, Polyethylene glycol 400, Sodium lauryl sulphate, Tween 20, Tween 60 and Propylene glycol) were used to prepare the gels. The best release profile of nystatin was obtained with 2% w/w nystatin, 5% w/w chitosan and 10% w/w propylene glycol and it occurs through fickian diffusion mechanisms. The effect of preparation method was also evaluated and it was found that a significant increment in nystatin release from the selected formula was obtained with 3% acetic acid. The viscosity of the prepared gels increases significantly with increasing chitosan concentration (p<0.05) and incorporation of nystatin into the gel has no significant effect on viscosity. In vitro microbiological study for antifungal activity of the selected formula was performed and compared with the commercially available nystatin suspension; results indicated that chitosan significantly enhanced the antifungal activity of nystatin against Candida albicans.

Keywords: Chitosan, Nystatin, Bioadhesive, Candidiasis.

INTRODUCTION

A major difficulty for the successful eradication of fungal infections of the oral cavity is the dilution and rapid elimination of topically applied drugs due to the flushing action of saliva. The delivery system in which the drug is incorporated is therefore an important consideration and should be formulated to prolong retention of the drug in the oral cavity.

Bioadhesive polymers have been utilized in gel forms to prolong the residence time on oral mucosa and also reduce the frequency of application and the amount of drug administered, which might improve patient compliance and acceptance (1).

Chitosan, β (1, 4)-2-amino-2-d-glucose, is a cationic biopolymer produced by alkaline N-deacetylation of chitin, which is the main component of the shells of crab, shrimp, and krill. Chitosan has found many biomedical applications, including tissue engineering, owing to its biocompatibility, low toxicity, and degradation in the body by enzymes such as chitosanase and lysozyme. This has opened up avenues for modulating drug release in vivo in the treatment of various diseases (2).

Various studies showed the potential of chitosan-based gel via different routes of administration such as: oral (3), ophthalmic (4), vaginal (5), transdermal (6), nasal (7), and buccal (8, 9) delivery.

Nystatin is a polyene antifungal antibiotic used for the prophylaxis and treatment of candidiasis of the skin and mucous membranes. Is produced by growth of certain strains of streptomyces noursei. It is characterized as very slightly soluble to practically insoluble in water, containing not less than 5000 units per mg of the dried...
The aim of this study is to investigate the possibility of nystatin antifungal activity enhancement in buccal cavity by using chitosan as a gelling agent through the formulation of nystatin as an oral gel and then the performance of biological in vitro evaluation for the prepared gel.

**MATERIALS AND METHODS**

The materials used in this investigation were: Nystatin powder (Roztoky Company, Czech Republic), chitosan with molecular weight $2.5 \times 10^3$ KD (Biotech Co. Ltd, Korea), glacial acetic acid, potassium dihydrogen orthophosphate, polyethylene glycol 400 (BDH Chemical Ltd Poole, England), sodium lauryl sulphate, glycerol (Searle Company, Hopkin and Williams, England), sodium hydroxide, propylene glycol, Tween 20, Tween 60, lactic acid (Merck - Schuchardt, Germany), methanol (Scharlau chemisbarshelona, Spain) and Sabouraud dextrose agar (Difco laboratories, USA).

**Preparation of the nystatin gel**

Nystatin gel was prepared by dispersion of nystatin powder 0.2 gm in glycerol (5% w/w) using a mortar and a pestle. Ten milliliters of Glacial acetic acid (3% w/w) was then added with continuous mixing and finally chitosan polymer was spread on the surface of the dispersion and mixed well to form the required gel. The strength of the prepared gel (10 gm) is 100,000 IU nystatin in each gm of the base.

Nystatin gel had been prepared with three different concentrations of chitosan gelling agent 5%, 6% and 7% w/w. Also different concentrations of nystatin (1%, 2% and 3% w/w) were used with 5% w/w chitosan to study the effect of nystatin concentration on its release from the base.

In addition, the effect of preparation method on nystatin release was studied using either 3% w/w glacial acetic acid or 3% w/w lactic acid. Each of these acids was added to the preparation to obtain the homogenous clear chitosan gel.

**Drug content**

A weight of 0.3 g sample was introduced in 1000 ml volumetric flask and stirred on magnetic stirrer for three hours. A portion of the solution was filtered through a Whatman filter paper No.4 and 1 ml of the filtrate was transferred to a 100 ml volumetric flask and completed to volume with distilled water. The absorbance of the solution was determined at a wave length of 305 nm using UV spectrophotometer (Cintra 5, GBC Scientific equipment, Australia) and the concentration of the drug in the sample was calculated using the slope and the intercept obtained from the standard curve of nystatin in distilled water.

**Determination of gel pH**

One gram of the prepared gels was accurately weighed and dispersed in 10 ml of purified water. The pH of the dispersions was measured using pH meter (HANNA instruments, HI8417, Portugal).

**In vitro study of drug release**

The release study was carried out with USP dissolution apparatus type 1, Copley U.K., slightly modified in order to overcome the small volume of the dissolution medium, by using 100 ml beakers instead of the jars.

The basket of the dissolution apparatus (2.5 cm in diameter) was filled with 1 gm of nystatin gel on a filter paper. The basket was immersed to about 1 cm of its surface in 50 ml of phosphate buffer pH 6.8, at 37ºC ± 0.5 and 100 rpm.

Samples (2ml) were collected at 0.25, 0.5, 0.75, 1, 2, 3, and 4 hours and were analyzed spectrophotometrically by U.V. Spectrophotometer (Cintra 5, GBC Scientific equipment, Australia) at $\lambda_{max}$ 306 nm. Each sample was replaced by the same volume of phosphate buffer pH 6.8 to maintain its constant volume and sink condition.

Also the effect of the type and the concentration of different solubilizing agents on nystatin release from 5% w/w chitosan gel was studied using glycerol, 5%, 10% and 15% w/w, polyethylene glycol 400, 2%, 3% and 4% w/w, Tween 20 2% and 6% w/w, Tween 60 2% and 6% w/w, sodium lauryl sulphate 1% w/w, and propylene glycol 10%, 20% and 30% w/w.

**Viscosity**

Viscosity measurements of gels were performed to
evaluate the effect of different chitosan concentration on the apparent viscosity using a Brookfield digital viscometer (Model DV-II, USA). The speed was set on 100 rpm and spindle S27 was selected\(^{17}\).

**In vitro microbiological study**

A strain of Candida albicans was isolated from infected patients in the medical laboratory of Sant Raphael hospital and identified using germ tube test (identification test for Candida albicans\(^{18}\)). The isolated fungi were sub-cultured on Sabouraud dextrose agar.

The effectiveness of the prepared nystatin gel against Candida albicans was studied, by applying 0.4 gm of the gel on the sabouraud dextrose agar which was previously seeded with Candida albicans. This was then incubated at 37ºC for 24 hours. The effectiveness of the prepared gel was compared with chitosan gel contains 0% of nystatin and the reference nystatin suspension contains 100,000 I.U. of nystatin / 1mL (Mikostat \(^{8}\), Julphar). The zones of growth inhibition were measured for all the tested samples. Each type of the samples was tested in triplicate.

**Statistical analysis**

Results are expressed as mean ± S.D for triplicate samples. The results were statistically analyzed significant differences among the groups were determined by one-way analysis of variance (ANOVA) using SPSS statistical analysis program. Statistical significant was considered at \(p<0.05\).

**RESULTS AND DISCUSSION**

**Properties of Nystatin-Chitosan Gel**

Nystatin content in 0.3 g of the different gel formulations from the prepared formulae was presented in table 2. The prepared gel formulations have uniform distribution of drug content, homogenous texture and yellow color. The pH of the formulations was ranging from to 5-6.5. The rapid decrease in nystatin antifungal activity observed with acid preparation confirm in published data\(^{19}\). The ranges of pH values recommended to optimize nystatin stability and antifungal efficacy are 5.0 to 7.0 and 6.0 to 8.0 respectively\(^{20}\).

Moreover, the presence of acid must be proscribed because it makes candidiasis growth easier\(^{21}\).

However, in our study, the pH ranges did not seem to alter nystatin stability and maintain its antifungal activity.

**Viscosity of nystatin gels**

The viscosity study was performed choosing 2% nystatin gels with different chitosan concentrations to evaluate the effect of the base on gel viscosity.

The 2% nystatin concentration was chosen since it gave the best *in vitro* release profile comparing with other nystatin concentrations, as discussed in the next section.

Table 1 represents the relation between the concentration of chitosan gels and viscosity (cp). It was found that the viscosity of the prepared gels increases significantly \((p<0.05)\) with increasing chitosan concentration. On the other hand, incorporation of nystatin into the gel has no significant effect on viscosity.

**In vitro release of nystatin**

The *in vitro* release of nystatin from chitosan gels was carried out using USP dissolution apparatus type I. As the regression analysis of the obtained results for two kinetic models including zero order and Higushi’s model showed that Higushi’s model gave the highest value of \(r^2\) with significant difference \((p < 0.05)\).

Higushi’s model, where the cumulative amount of the released drug per unit area is proportional to the square root of time, is more suitable model to describe the release kinetics of nystatin from the gel preparations examined in the present study. Higushi’s rate constants were calculated and summarized in table 1.

<table>
<thead>
<tr>
<th>Chitosan (w/w %)</th>
<th>Nystatin (w/w %)</th>
<th>Viscosity (cp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5%</td>
<td>0</td>
<td>1300 ± 2.51</td>
</tr>
<tr>
<td>5%</td>
<td>1</td>
<td>1297 ± 1.64</td>
</tr>
<tr>
<td>5%</td>
<td>2</td>
<td>1298 ± 1.76</td>
</tr>
<tr>
<td>5%</td>
<td>3</td>
<td>1298 ± 0.53</td>
</tr>
</tbody>
</table>

\(^{*}p<0.05\)
To study the effect of chitosan base concentrations on nystatin release profile, a model formula had been chosen (2%w/w nystatin, 5%w/w glycerol with 5, 6 and 7% w/w chitosan). It was found that, nystatin release affected significantly ($p < 0.01$) by the concentration of chitosan base and ranked as: 7%< 6%< 5% w/w, i.e. there was an inverse relationship between chitosan concentration and the release rate constant, as shown in Figure 1. The highest drug release constant was obtained with 5% w/w chitosan base (table 2).

**Figure 1.** Effect of chitosan concentration on nystatin release in phosphate buffer pH 6.8.

**Table 2:** Drug Content and Higuchi’s Rate Constant (K) ± SD of Nystatin for Gel Formulations.

<table>
<thead>
<tr>
<th>Enhancers</th>
<th>Drug Content (%)</th>
<th>Chitosan (5% w/w)</th>
<th>Chitosan (6% w/w)</th>
<th>Chitosan (7% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol 0%</td>
<td>101.2 ± 1.54</td>
<td>0.0187 ± 0.001 ($r^2 = 0.973$)</td>
<td>0.0305 ± 2.0 ($r^2 = 0.948$)</td>
<td>0.0230 ± 2.7 ($r^2 = 0.983$)</td>
</tr>
<tr>
<td>Glycerol 5%</td>
<td>99.5 ± 1.36</td>
<td>0.0370 ± 0.027 ($r^2 = 0.983$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerol 10%</td>
<td>97.3 ± 2.43</td>
<td>0.0419 ± 0.032 ($r^2 = 0.959$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerol 15%</td>
<td>99.3 ± 0.91</td>
<td>0.0571 ± 0.0079 ($r^2 = 0.971$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEG 2%</td>
<td>98.7 ± 0.87</td>
<td>0.0412 ± 0.009 ($r^2 = 0.971$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEG 3%</td>
<td>100.1 ± 2.12</td>
<td>0.0409 ± 0.019 ($r^2 = 0.905$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEG 4%</td>
<td>99.7 ± 1.26</td>
<td>0.0401 ± 0.093 ($r^2 = 0.968$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLS 1%</td>
<td>98.3 ± 2.98</td>
<td>0.0405 ± 0.03 ($r^2 = 0.961$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T20 2%</td>
<td>99.6 ± 1.75</td>
<td>0.0712 ± 0.02 ($r^2 = 0.958$)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
These results may suggest that, drug release from chitosan gel depends on the physical network structure (open cell like structure). Drug release occurs through the pores of the low polymer concentration while chitosan concentration increment resulted in more cross-linking of the network structure; consequently slower drug release from the gel base was achieved (22, 23).

Also increasing the polymer concentration in the gel increases viscosity which prolonged drug diffusion through the gel matrix therefore reduces nystatin release rates from the gels. The same effect was obtained by Attia et al (1) who studied the diffusion of piroxicam from different polymer gel at different concentrations of (sodium alginate 7%, 10% w/v, hydroxypropylmethylcellulose 2.5%, 5% w/v and methyl cellulose 3%, 5% w/v). Moreover, lidocaine diffusion from poloxamer 407 gel decreases with increment of poloxamer content (24).

Figure 2. Release profile of nystatin 2% from chitosan gel, using two different methods of preparations in phosphate buffer pH 6.8.
In addition, a high significant enhancement (p<0.01) in the nystatin release constant was obtained with glacial acetic acid rather than with the addition of lactic acid (Figure 2). These findings may be explained on the existence of hydroxyl groups in addition to carboxyl groups in lactic acid structure that are responsible for further interaction with chitosan base than acetic acid through H-bonds formation, resulting in more cross-linking and less porous network structure of chitosan gel. Consequently less drug release from lactic acid than acetic acid-chitosan gel was yield.

The release of nystatin from chitosan gel 5% was studied with different nystatin concentrations (1, 2 and 3 %w/w), as shown in Figure 3. It can be observed that, a significant increment in percentage of drug release (p<0.05) from the gel was obtained with 2%w/w than 1 %w/w of nystatin. This result was in agreement with the research done by Sang et al (25), who found that, triamcinolone acetonide release from carbopol and poloxamer 407 gels was increased with increment of its concentration from 0.05 to 0.1 %. While further increase in nystatin concentration (3%) resulted in a decrease in its release from the gel. This may be due the capability of nystatin to interact with different polymers. Gavrilin et al (26) proved that nystatin ointment based on polyethylene oxide (PEO) 1500 and PG is superior to the existing commercial prototype with respect to antifungal activity. The increase in activity is due to the fact that nystatin in the ointment occurs in the dissolved state and forms interaction products with PEO. The principal mechanism of such interactions is the formation of hydrogen bonds involving amino group and hydroxy groups of nystatin.

In our study further future investigations are required to prove the type and the proposed mechanism of nystatin-chitosan interaction.

The effect of different solubilizing agents and their concentrations on drug release was studied. Table 2 shows the effect of glycerol concentrations (0%, 5%, 10% and 15% w/w) on nystatin release from 5% chitosan gel. It was found that increasing the loading concentration of glycerol in the gel from 0% to 15% resulted in a high significant increase (p < 0.01) in drug release constants.

This result is consistent with other studies that had been done in this field. Glycerol was found to enhance the
solubility of valdecoxib \(^{(27)}\) by decreasing the dielectric constant of the cosolvent-water mixtures. Also The solubility of rofecoxib increased with increasing mass fraction of cosolvents including ethanol, glycerol propylene glycol to different extent\(^{(28)}\). Furthermore Darwish\(^{(29)}\) found that, the addition of glycerol as co-solvent to the paraben solutions produced a decrease in the o/w partition coefficient and enhanced the efficacy of the parabens in oil/water formulations. Accordingly glycerol may enhanced the solubility of nystatin by decreasing its hydrophobicity and consequently increase the drug release from the gel.

![Figure 4. Effect of different concentrations of propylene glycol on nystatin release from chitosan gel in phosphate buffer pH 6.8.](image)

On the other hand, the addition of polyethylene glycol (PEG 400) and sodium lauryl sulphate (SLS) 1\% to the gel showed no significant effect on drug released, as shown in Table 2.

However, Tween 20 and Tween 60 showed a good enhancement of nystatin release from 5\% w/w chitosan gel, as represented in Table 2. But the addition of higher concentration (6\%) of these solubilizers to the formula resulted in a significant (\(p < 0.05\)) decline in the release constant of nystatin from chitosan gel. This may be attributed to the solubility of the drug in the gel rather than the media.

There are many reports demonstrated an increase in drug release due to the addition of surfactants \(^{(30-33)}\). Solubilization of water insoluble drugs by micelles has long been investigated as a means of improving solubility for drug delivery \(^{(34)}\) and the incorporation of a wide variety of drugs into micelles formed from a large variety of surfactants in particular non-ionic surfactants have been studied \(^{(35, 36)}\). Andersen et al\(^{(27)}\) found that nystatin release from a chewing gum formulation as drug delivery device with addition of non-ionic surfactants Tween 60 promoted a far higher release of nystatin, in view of that, our results are consistent with these findings.
Table 3. Release Exponent (n) and correlation coefficient (r²) for the Prepared Chitosan Gels.

<table>
<thead>
<tr>
<th>Chitosan Gels</th>
<th>n</th>
<th>r²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitosan 5%</td>
<td>0.461</td>
<td>0.954</td>
</tr>
<tr>
<td>Chitosan 6%</td>
<td>0.557</td>
<td>0.976</td>
</tr>
<tr>
<td>Chitosan 7%</td>
<td>0.541</td>
<td>0.966</td>
</tr>
<tr>
<td>Glycerol 0%</td>
<td>0.582</td>
<td>0.958</td>
</tr>
<tr>
<td>Glycerol 5%</td>
<td>0.368</td>
<td>0.993</td>
</tr>
<tr>
<td>Glycerol 10%</td>
<td>0.248</td>
<td>0.983</td>
</tr>
<tr>
<td>Glycerol 15%</td>
<td>0.404</td>
<td>0.972</td>
</tr>
<tr>
<td>PEG 2%</td>
<td>0.420</td>
<td>0.964</td>
</tr>
<tr>
<td>PEG 3%</td>
<td>0.458</td>
<td>0.949</td>
</tr>
<tr>
<td>PEG 4%</td>
<td>0.620</td>
<td>0.983</td>
</tr>
<tr>
<td>SLS 1%</td>
<td>0.785</td>
<td>0.987</td>
</tr>
<tr>
<td>T20 2%</td>
<td>0.555</td>
<td>0.937</td>
</tr>
<tr>
<td>T20 6%</td>
<td>0.504</td>
<td>0.921</td>
</tr>
<tr>
<td>T60 2%</td>
<td>0.516</td>
<td>0.948</td>
</tr>
<tr>
<td>T60 6%</td>
<td>0.549</td>
<td>0.916</td>
</tr>
<tr>
<td>PG 10%</td>
<td>0.470</td>
<td>0.986</td>
</tr>
<tr>
<td>PG 20%</td>
<td>0.713</td>
<td>0.961</td>
</tr>
<tr>
<td>PG 30%</td>
<td>0.717</td>
<td>0.995</td>
</tr>
</tbody>
</table>

Finally, the best nystatin release from chitosan gel was obtained with the use of 10% of propylene glycol (PG), as illustrated in Figure 4. These results are consistent with other studies who found that the final viscosity of gel formulation is directly related to PG concentration (38, 39). Consequently, using 30% PG resulted in a significant decrease in drug release from the gel base due to the increase in gel viscosity.

The amount of nystatin released from chitosan gels, for all the tested time intervals, was found to be greater than the reported nystatin MIC (0.14 µg/ml) against Candida albicans (40).

Further treatment was done for the release data obtained with the prepared chitosan gels to pick up the best fit mechanism of drug diffusion from the film. The release data was fitted to the following equation (41):

\[ F = \frac{M_t}{M_{\infty}} = K t^n \quad \ldots \ldots \quad (1) \]

Where \( M_t \) is the amount released at time \( t \), \( M_{\infty} \) is the initial amount of drug, \( K \) is a constant incorporating structural and geometrical characteristics of the control release device, \( n \) is the release exponent which indicates the mechanism of drug release and \( F \) is the fractional release of drug. When \( n \) equals to 0.5 the mechanism is fickian...
transport, 0.5-1 it is non-fickian transport. If it is equal to zero, so mechanism is zero order transport.

By taking ln of equation (1), the following expression can be obtained:

\[ \ln \frac{M_t}{M_\infty} = \ln K + n \ln t \] \hspace{1cm} (2)

Straight lines were obtained from plotting of \( \ln \frac{M_t}{M_\infty} \) vs. \( \ln t \) and the kinetic parameter \( n \) was calculated, with the correlation coefficients \( r \) as shown in table 3. Results indicate that, the release of nystatin from the prepared chitosan gels is fickian transport except for those containing SLS 1%, PEG 4%, PG 20% and 30%, where \( n \) is more than 0.5. This may indicates that the release of nystatin from chitosan gel base with presence of high concentration of these solubilizing agents occur through both erosion and fickian diffusion mechanisms.

**In vitro microbiological study**

Candida albicans was susceptible to the blank chitosan gel containing 0% nystatin as well as to the selected formula (5% w/w chitosan gel, 2% w/w nystatin and 10% w/w PG) and the commercial suspension (Mikostat®, JULPHAR). Growth inhibition zones ± SD of Candida albicans by the addition of Mikostat® suspension, blank gel and nystatin-chitosan gel were 26.7±0.5 mm, 19.6±0.7 mm and 32.2±0.8 mm respectively. This indicates that a significant \( (p<0.05) \) enhancement of nystatin antifungal activity was obtained by a new formulation utilizing chitosan polymer as gelling agent.

Our results are in agreement with those obtained by Senel et al (9) who prepared chitosan films and hydrogels of chlorhexidine gluconate for oral mucosal delivery and studied chitosan inhibition effect on growth of Candida albicans.

**CONCLUSION**

The present study demonstrates that chitosan polymer is a candidate gelling agent for development of nystatin gel which can be used successfully for eradication of fungal infections in oral cavity. It was found that, maximum release of nystatin was obtained with 5% w/w chitosan gelling agent, acetic acid and 10 %w/w PG as solubilizing agent. In addition, increment of nystatin concentration resulted in more enhancement of drug release, while higher concentration of nystatin caused further decrease and retardation in its release from chitosan base. The release of nystatin from chitosan base with the presence of different types and concentrations of solubilizing agents occurs through both erosion and fickian diffusion mechanisms.

Moreover, in vitro microbiological study proved the enhancement of nystatin antifungal activity by its incorporation in chitosan gel, in comparison with the commercial reference product.

**REFERENCES**


In Vitro Release… Bazigha K. Abdul Rasool et al.

The study aimed to investigate the effect of various concentrations of vitamin A, vitamin D, and vitamin E on the release of kites from the female reproductive tract in the presence of external fluid and itsby.

Keywords: Female reproductive tract, vitamin A, vitamin D, vitamin E.