FORMULATION AND STABILITY TESTING OF GENTAMICIN-N. SATIVA FUSION EMULSIONS FOR OSTEO-HEALING APPLICATION

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ABSTRACT

An alternative osteo-healing formulation with osteo-healing properties was formulated by combining gentamicin and Nigella sativa (N. sativa) oil in an emulsion to reduce gentamicin toxicity effect over prolonged use in osteo-infection treatment. This work aims to test the aqueous solubility and physicochemical properties of the emulsion. Four emulsions (emulsion A, B, C and D) had been formulated, with final concentration of gentamicin was made constant at 0.1% (w/v) whereas N. sativa oil concentration was varied between 32.5% (v/v) to 46.4% (v/v) in all formulations. Then, stability studies of all emulsion were performed by centrifugation at (5000rpm, 5 minutes), at different storage conditions (8°C, 25°C and 50°C), organoleptic characteristics, freeze-thawcycle, pH determination, particle size measurement, zeta-potential analysis, and pH titration analysis. Results showed no phase separation after centrifugation for freshly prepared emulsions. Storage at 8°C, all emulsions also showed no phase separation at all-time points. At 25°C storage condition, three formulations were stable at day 7 but phase separation was formed in all emulsions by day 14 showed good stability at day 7 and all emulsions formed phase separation at day 14. No emulsions were stable in storage temperature of 50°C. The particle size of the emulsions increased with an increment of N. sativa oil concentration. Zeta-potential analysis showed a range of -32.2 ± 0.15 mV to -46.0 ± 0.45 mV. When pH titration analysis was performed, the zeta potential indicated that the emulsion stability was affected by acidic conditions. We concluded that the use of gentamicin-N. Sativaeumulsions must take into account the storage condition with preference of low temperature and fresh preparation at higher alkalinity and the lowest possibility of N. sativa oil.

Keywords: Gentamicin, Nigella sativa, Emulsion, Stability, Osteo-healing.

INTRODUCTION

The usage of gentamicin as a treatment for musculoskeletal infection locally or systemically has become increasingly popular. Local antibiotic delivery system has been used in the treatment of bone and tissue infection, either to supplement or to replace the use of systemic antibiotics [1]. However, antibiotic treatment in patients with poor vascularised infected tissues and osteonecrosis may be inadequate or ineffective. A long-term course of antibiotic therapy is a must but there is the side effect or toxicity over these prolonged therapies [1,2]. Therefore, gentamicin and Nigella sativa (N. sativa) oil was fused together to lower the toxic effect of gentamicin. N. sativa is known as black seed or black cumin and had been used in herbal medicine all over the world for the treatment and prevention of diseases and conditions [3,4,5] such as, decreases DNA damage, prevents initiation of carcinogenesis in colonic tissue [4] and effect against osteoporosis [6]. N. sativa has an active compound named thymoquinone, which has antioxidant activity and was derived from the fatty acid constituents present in the seeds. It is believed that the wound healing process due to thymoquinone may be effective in accelerating new bone formation and pH determination, particle size measurement, zeta-potential analysis and pH titration analysis were done to determine stability of emulsions.

MATERIALS AND METHODS

Materials

All the chemicals used in this study were of analytical grade; gentamicin sulphate powder purchased from local pharmacy, N. sativa oil purchased from Hemani Trading, Pakistan, sorbitan monolaurate (Span®20) purchased from Sigma (Sigma-Aldrich Co., USA), and PEG-20 sorbitan monolaurate (Twee®20). Distilled water was used for the preparation of emulsions.

Methods

Emulsification process

Gentamicin-N. sativa emulsions were formulated (Emulsion A, B, C & D) (Figure 1). Twee®20 and Span®20 were used as surfactant and co-surfactant respectively. Concentration of gentamicin was made constant at 0.1% (w/v) whereas the final concentration of N. sativa was varied between 32.5% (v/v) to 46.4% (v/v) in all formulations.
During the preparation of the emulsions, the solution was agitated slowly using a magnetic stirrer (Daihan Labtech, India) at a speed of 1500 rpm. Gentamicin sulphate powder was diluted in distilled water, followed by adding Tween®20 and Span®20, as surfactant and co-surfactant. N. sativa oil, then added and stirred thoroughly for 5 minutes (1500 rpm). The emulsions were homogenised using T10 basic Ultra-Turrax® (Germany) homogeniser for 5 minutes at 10,000 rpm.

Stability tests

The stability tests were performed at different storage conditions for gentamicin-N. sativa emulsions. The samples were kept at 8 ± 0.2°C (in refrigerator), 25 ± 0.3°C (in temperature-controlled room) and 50 ± 0.2°C (in oven) under different durations; day 0, 7, 14 and 30 respectively. The samples were then examined for their stability by properties of their organoleptic characteristics, centrifugation tests, particle size measurements, and pH readings.

Organoleptic characteristics

Stable formulation of gentamicin-N. sativa emulsions were prepared freshly and investigated organoleptically through the properties of colour, odour, texture and phase separation of the samples. The organoleptic characteristics of the emulsions kept in distinct storage conditions were observed and recorded at various intervals of day 0, 7, 14 and 30.

Centrifugation test

Centrifugation tests were performed for the gentamicin-N. sativa emulsions immediately after preparation. The same test was repeated after 7, 14 and 30 days of preparation. Centrifugation conditions were 25°C and 5000 rpm (5 minutes).

Freeze-thaw cycle

Three test samples and a control sample of gentamicin-N. sativa emulsions were prepared in four separate micro-centrifuge tubes. Initial observations were made for all samples. The test samples were placed in a freezer (-20°C) for 24 hours and then removed to be allowed to thaw at room temperature for 24 hours. The test samples were then put into an oven with temperature 50°C and left for 24 hours.

The test samples were then removed and equilibrated to room temperature for 24 hours. End observations were recorded based on the notability for any signs of phase separation in the test samples. This completes one cycle for the freeze-thaw cycle test. Another two cycles were repeated on the test samples to attain a good degree of confidence in the stability of the emulsions ([19]).

pH determination

Initial pH values of freshly prepared gentamicin-N. sativa emulsions were measured with a calibrated digital pH meter. The pH values of the emulsions kept at different storage conditions were also measured after each interval of day 0, 7, 14 and 30. Electrode of the digital pH meter was immersed directly into the emulsion during measurement. Triplicates were done in all pH measurements and the mean readings were recorded.

Particle size measurement

ZEN1600 Nano Particle Size Analyzer (Malvern Instruments, UK) was used to measure particle size of gentamicin-N. sativa emulsions. Ratio 1:1000 of the sample diluted with distilled water. The measurements of the particle size were done in triplicates and the mean results were recorded.

Zeta-potential analysis

The measurement of zeta-potential was measured at 25°C by using Malvern Zetasizer 4 (Malvern Instruments, UK) following 1:1000 dilutions in distilled water. The measurements of zeta-potential were done in triplicates and the mean results were recorded.

pH titration analysis

The measurement of pH titration was measured at 25°C by using Malvern Zetasizer 4 (Malvern Instruments, UK) following 1:1000 dilutions in distilled water. Measurements of pH titration were done in triplicates and the mean results were recorded.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Composition (% v/v)</th>
<th>Gentamicin (% w/v)</th>
<th>Observable Phase Separation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled Water</td>
<td>N. sativa Oil</td>
<td>Tween 20</td>
<td>Span 20</td>
</tr>
<tr>
<td>A</td>
<td>38.9</td>
<td>32.5</td>
<td>23.4</td>
</tr>
<tr>
<td>B</td>
<td>37.5</td>
<td>35.0</td>
<td>22.5</td>
</tr>
<tr>
<td>C</td>
<td>34.5</td>
<td>40.2</td>
<td>20.7</td>
</tr>
<tr>
<td>D</td>
<td>30.9</td>
<td>46.4</td>
<td>18.6</td>
</tr>
</tbody>
</table>
Further investigations regarding stability of A, B, C and D showed no phase separation formed at day 0, 7, 14 and at the end of storage conditions (Table 2). The result at 8°C, emulsion 30. At 25°C, all emulsions started to form phase separation at day 14 onwards. At 50°C, all emulsions formed phase separation at day 7 and onwards. Gravitational force acts in the emulsions generate sample stress and thus increase the particle mobility and started to produce instabilities. Therefore, phase separation was formed after stored in certain storage conditions and durations [14].

**Table 3: Organoleptic characteristic observation of gentamicin- N. sativa emulsions (Emulsion A, B, C and D) stored at different storage conditions, observed at day 0, 7, 14 and 30.**

<table>
<thead>
<tr>
<th>Duration (Day)</th>
<th>Color</th>
<th>Phase Separation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8°C</td>
<td>25°C</td>
</tr>
<tr>
<td>Day 0</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>Day 7</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>Day 14</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>Day 30</td>
<td>M</td>
<td>M</td>
</tr>
</tbody>
</table>

-= No Change, += Slight change, ++= More change

**RESULTS AND DISCUSSION**

Formulation gentamicin-N. sativa emulsions

Gentamicin-N. sativa emulsion A, B, C and D were formulated with a constant concentration of gentamicin sulphate (0.1% (w/v)) whereas N. sativa oil concentration was ranging from 32.5% to 46.4% (v/v) (Table 1). Further investigations regarding stability of the emulsions were tested in stability tests.

**Stability tests**

Centrifugation test

Centrifugation test is done to determine the behaviour of an emulsion at the end of storage conditions (Table 2). The result at 8°C, emulsion A, B, C and D showed no phase separation formed at day 0, 7, 14 and 30. At 25°C, all emulsions started to form phase separation at day 14 and onwards. At 50°C, all emulsions formed phase separation at day 7 and onwards. Gravitational force acts in the emulsions generate sample stress and thus increase the particle mobility and started to produce instabilities. Therefore, phase separation was formed after stored in certain storage conditions and durations [14].

**Organoleptic characteristics**

Organoleptic characteristics are defined as an observation of the appearance of emulsions by its colour, odour, formation of phase separation, precipitation, turbidity and many more [13]. The observation results as in Table 3. The colour of all stable emulsions was milky white (Figure 2). The emulsion smells like N. sativa oil and the texture emulsions were sticky. No phase separation was seen at temp 8°C and the colour of emulsions remains milky white. At 25°C phase separations formed at day 7 for emulsion A and at day 14 for emulsion B, C & D. At temperature 50°C, all emulsions changed colour to golden brown and phase separation formed from day 7 onwards. Colour, odour and texture of the emulsion should be unaltered over time [13]. Thus, emulsion must be stored at 8°C to its colour, odour and texture.

**Freeze-thaw cycle**

Freeze-thaw cycles, evaluate the stability of emulsions by challenging temperature shock that the product could suffer, which cause problems such as phase separation, crystal formation, rheological properties damaged and etc. [14]. Results (Table 4) showed that all emulsions became unstable when challenged with extreme temperature. This may be influenced by polymorphism, degree of lipid crystallinity and phase behaviour of water. When emulsion freeze, lipid droplets become concentrated into freeze-concentrates phase. Therefore coming in close contact with one another in the unfrozen aqueous channel between crystals. The concentration of the lipid droplets in these narrow channels could promote aggregation, flocculation or coalescence during the freeze-thaw process [15,16]. Hence, emulsion must be kept away from extreme conditions and huge fluctuations of temperature.

**Table 4: Freeze-thaw cycle results of freshly prepared gentamicin-N. sativa emulsions (Emulsion A, B, C & D) for three cycles.**

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Emulsion A</th>
<th>Emulsion B</th>
<th>Emulsion C</th>
<th>Emulsion D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cycle 1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cycle 2</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Cycle 3</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>
pH determination

pH value for all freshly prepared emulsions were between pH 5.73 to 5.79 (Figure 3). When stored at 8°C, the pH increased in all emulsions while at ≥25°C storage temperature, pH decreased in all emulsions. Emulsions stored in 50°C showed a fluctuation of pH values. This is because, at high temperature, physicochemical parameters were altered since the temperature elevation possesses direct influence on stability of pharmaceutical dosage forms and the active substance in *N. sativa* oil. Furthermore, low pH emulsions showed poor stability compared to emulsions with higher pH [17]. Increasing of pH could be related to volatile aldehyde oxidation to carboxylic acids or lipid enzymatic hydrolysis that release free fatty acids contains in *N. sativa* oil [18].
**Particle size measurement**

Particle size of emulsions increased with an increment of *N. sativa* oil concentration (Figure 4). This may be due to increase fatty acid from *N. sativa* oil. Particle sizes of freshly prepared emulsions were ranging from 800 to 1296 nm. In a well-formulated emulsion, the droplet size would be between 1 to 5 micro-meters. At 8°C, particle size of the emulsions became smaller may be due to the attractive forces acting between droplets decreased while inversely, at 25°C and 50°C, the size of particles increased. Sometimes particle size does not affect the stability of the emulsions. Some emulsions with large particles, which were greater than 10 micro-meters showed good long-term stability [19]. But most of the time, attractive forces acting between droplets decrease with smaller particle size and will give better stability against droplet flocculation and coalescence. Previous studies have shown that using a high energy approach (such as homogenizer type, speed, temperature and time), sample composition (such as oil type, surfactant type and relative concentration) and physical properties of component phases (interfacial tension and viscosity) [20]. Thus, homogenizer can be used to achieve minimum particle size so that the stability, appearance, texture and bioavailability of good emulsions can be produced [21,22].

**Zeta-potential analysis**

The zeta-potential of all emulsions were lower than -30mV and became more negative as the *N. sativa* oil increased (Figure 5). The negative charged emulsion was contributed by negative charged surfactants, which are also known as anionic surfactants. The surfactants used were Tween®20 and Span®20 which were categorised as soap surfactants [19]. Additionally, emulsifiers and surfactants can be classified as cationic (positively charged), anionic (negatively charged), amphoteric or zwitter ionic (both positively and negatively charged) and non-ionic (no-charged). Most of emulsifiers and surfactants were amphiphilic molecules which consist of hydrophobic and hydrophilic parts. Polar group of charged substances attached to hydrocarbon chain and exhibit both hydrophobic and hydrophilic characteristics [23]. The influence of the surfactants of the emulsion characteristics was demonstrated that the surface charge might affect physical and chemical stability [24,25]. Thus, it could be considered that all emulsions prepared had good stability and repulsive forces between droplets were larger in the emulsions containing oil concentrations.

![Fig. 5: Measurement of zeta-potential of gentamicin-*N. sativa* emulsions (Emulsion A, B, C and D) stored at different storage conditions, measured at day 0, 7, 14 and 30 (n=3).](image)

![Fig. 6: pH titration range and zeta-potential (mV) of freshly prepared gentamicin-*N. sativa* emulsions (Emulsion A, B, C and D) (n=3).](image)
pH titration analysis

The pH titration of freshly prepared emulsions (Figure 6) showed that the emulsion stability was affected by acidic conditions but not by alkaline conditions. Unstable emulsion can be identified by the emulsion stability was affected by acidic conditions but not by particles become protonated when adsorb at oil-water interface and prepared at high temperature then decreased progressively. The demulsification process, which can be occurred when emulsion detached from the interface in-situ. Thus, the degree of ionisation of emulsion particles was taken into consideration in controlling the coalescence stability of emulsions [26].

CONCLUSION

Although the tools for characterising emulsions are advanced and mechanisms of emulsification understood, it is still difficult to predict the actual results of an emulsification process, because many parameters involved. The emulsions showed that storage temperature does affect the stability of emulsions. Extreme temperature must be avoided during storage of emulsion to maintain the stability. Thus, understanding the role of each parameter can aid in designing the stable gentamicin-N. sativa emulsions.

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CONFLICT OF INTERESTS

Declared None

REFERENCES