Formulation of Heterolipid-Based Hollow Nanoparticles Improves the Physicochemical and Gastroprotective Properties of Cimetidine

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Abstract: Purpose: The purpose of the present study is to improve the physicochemical and gastroprotective properties of the BCS class III drug, cimetidine using nanostructured lipid carriers (NLC); thus, improving the overall absorption and bioavailability of cimetidine.

Methods: Cimetidine-loaded NLCs were formulated by melt-fusion followed by high pressure homogenization (HPH) using rational blends of the solid lipids, Precirol® ATO5 and beeswax, and the liquid lipid, Kolliphor® ELP. In vitro physicochemical characterization (particle size, electric charge, polydispersity index, thermal analysis, particle optics, entrapment efficiency) and in vivo gastroprotective evaluations were carried out respectively.

Results: Cimetidine-loaded NLCs were stable (~31 to -37 mV), polydispersed (0.1–0.4) with particle sizes ranging from 113 – 153 nm. Thermal analysis confirmed that cimetidine was molecularly dispersed in the NLC due to low matrix crystallinity. In vitro release of cimetidine showed sustained release effect and entrapment efficiency (EE %) was concentration-dependent in the range of 85 – 96 %. There was improved gastric ulcer inhibition by the cimetidine-loaded nanoparticles.

Conclusion: The improved physicochemical and gastroprotective profiles obtained from the study suggest that NLC is a very promising delivery system for cimetidine.

Keywords: Cimetidine, Precirol® ATO5, Beeswax, Gastroprotective, Nanostructured lipid carrier.

INTRODUCTION

Peptic ulcer disease (PUD) including gastric and duodenal ulcers, is an important GI disorder which occurs as a deep and severe injury or lesion in the GIT. Depending on the depth of the lesion, PUD might erode the muscularis mucosae due to its aggressive negative effect on the mucosal defenses. It is often characterized by burning sensation in the epigastrium, belching, nausea, vomiting, oedema, bleeding, scarring, gastric perforation, haemorrhage, and gastric outlet obstruction [1-3]. The exact aetiology of PUD is not clear, but it is reportedly caused by factors like hyperacidity in the gastric mucosa, imbalance among the aggressive acid-pepsin secretions and impaired mucosal resistance, infection by Helicobacter pylori, and misuse of non-steroidal anti-inflammatory drugs (NSAIDs). It has also been reported that hereditary conditions could lead to ulcers but the mechanism is not yet clear [4, 5]. Cimetidine (N-cyano-N-methyl-N-[2][(5-methyl-1H-imidazol-4-yl) methyl] thio] ethyl] guanidine is the first drug developed for the clinical treatment of PUD, dyspepsia and gastroesophageal reflux disease (GERD) by GlaxoSmithKline and marketed as Tagamet® [6]. According to the biopharmaceutical classification system (BCS), cimetidine is a class III drug, and drugs in this class account for about 25 % of drugs used in the US, and about 40 % of drugs listed in the World health Organization (WHO) List of Essential Medicines [7, 8]. Generally, drugs in this class have high solubility and low permeability. However, cimetidine is sparingly soluble (11.4 mg/ml at 37 °C), undergoes incomplete absorption following oral administration with low intestinal permeation. Consequently, the incomplete solubilization and absorption of cimetidine, especially in PUD, lead to unpredictable bioavailability and irreproducible clinical response or outright therapeutic failure [9].

Recently, the development and introduction of lipid nanoparticles as an acceptable alternative delivery system to tablets and capsules for solving incomplete
dissolution, absorption and bioavailability challenges affecting drugs (lipophilic and hydrophilic) has become a very interesting research subject [10, 11]. Essentially, nanostructured lipid carriers (NLC) were introduced as a recent and attractive technique widely applied by formulation scientists to improve the physicochemical, pharmacokinetic (PK) and biodistribution (BD) profiles of drugs [12-14]. NLC consist solid lipid and liquid lipid (oil) in which the oil is localized in the core of the nanoparticles and solubilizes the drug resulting in high drug loading capacity, improved encapsulation efficiency and drug solubilization, better absorption and controlled drug release. The presence of the oil in the solid lipid matrix avoids drug crystallization, drug expulsion, and creates numerous imperfections in the lipid carrier system to encapsulate a high amount of the drug due to molecular rearrangement in the lattice structure of the lipid core, and stabilize the nanoparticles [15-17]. Therefore, for the first time, NLC of cimetidine was developed, characterized for improved dissolution and absorption, and the gastroprotective property of the NLC was evaluated in vivo.

MATERIALS AND METHODS

Materials

The following materials were used as procured without further treatment: Cimetidine was a kind gift from Pauco Pharmaceuticals, Awka, Nigeria. White beeswax (BW) was a gift from Cera Alba Pellets, Hilden, Germany. Precirol®ATO5 (PATO5) was kindly provided by Gattefosse, Saint-Priest Cedex, France. Softisan®154 (S154) was donated by Fa.Condea Chemie GmbH, D-58453Witten, Germany. Kolliphor®ELP (KELP) and Kolliphor®P188 (KP188) were kindly provided by BASF SE, Ludwigshafen, Germany. The brand of commercially available cimetidine used was Tagamet® (GlaxoSmithKline, USA). Distilled water was obtained from an all-glass still. All other chemicals and reagents used were commercially obtained and of analytical grade.

Screening of Formulation Ingredients

Selection of Lipid Excipients

Binary mixtures of each solid and liquid lipid were prepared (Precirol®ATO5-Kolliphor®ELP at 1:1, Beeswax-Kolliphor® ELP at 2:1, and Softisan®154-Kolliphor® ELP at 1:2) respectively by melting the solid lipid at 90 °C and mixing with 10 ml of the oil at 100 rpm (Magnetic stirrer, Jenway, Bibby Scientific, Essex, UK) for 3 h. After blending, the lipid matrices were allowed undisturbed for 48 h to cool at room temperature (27 ± 2 °C) for complete recrystallization and each matrix was stored in an air-tight plastic container until used. Next, the solid lipids were mixed with each other (PATO5-BW, PATO5-S154, S154-BW) at the above ratios for modification of the crystal profiles of the solid lipids. Finally, ternary lipid systems comprising a blend of two solid lipids mixed with the oil (PATO5-BW-KELP, PATO5-S154-KELP, and S154-BW-KELP) were prepared as above and stored. The two binary lipids and the ternary lipid matrices as well as the bulk lipids were studied with differential scanning calorimeter (DSC 204 F1 Phoenix, Netzsch, Germany) to ascertain their thermal behaviour and suitability for formulation of NLC. The lipid matrix with the least enthalpy was considered for assay of drug solubility because low enthalpy suggests low crystallinity and better chances of high drug entrapment [12].

Drug Solubility and Crystallinity Evaluations

To ascertain the suitability of the selected lipid matrices for the formulation of NLC, 1 g each of the selected ternary matrix was melted at 90 °C at room temperature, and amounts of cimetidine (0.1 and 0.5 g) were added to the molten lipids respectively with stirring at 100 rpm (Jenway, Bibby Scientific, Essex, UK). The miscibility and solubility of cimetidine in the molten lipids was assessed visually every 20 min observing the presence or absence of drug crystals during melting and mixing, and upon cooling at room temperature. Solubility of cimetidine in the liquid lipid and a dispersion of the surfactant were also carried out, but this assessment was limited to visual observation. Furthermore, the surfactant and the oil were mixed and their interaction was visually observed. Thereafter, the drug-loaded lipid melts were investigated using the same DSC as above to determine if recrystallization of cimetidine occurred from the molten lipids. The thermal properties of cimetidine alone were also determined using DSC [12, 14].

Scanning Electron Microscopy (SEM)

The morphology of the cimetidine-loaded and unloaded ternary lipid matrix of choice was determined using Phenom ProX scanning electron microscope (PhenomWorld, Eindhoven, Netherlands). The samples prepared from dispersion in bidistilled water were placed on the sample holder of the scanning electron
microscope and the temperature of the sample chamber set to – 5 °C to freeze-fracture the sample. After 5 min allowed for instrument stabilization, sample imaging was carried out at an accelerated voltage of 15kV and 2000× magnification. Sample images were focused using a NavCam digital camera, and transferred to Phenom suite software for particle morphology analysis and fibrometric or pore measurements [18].

**Formulation of Cimetidine-Loaded Nanodispersions**

Nanostructured lipid carriers (NLC) of cimetidine were prepared by melt-fusion method [10] followed by high pressure homogenization using quantities of ingredients as shown in Table 1. First of all, ternary lipid matrices were prepared at ratios of 1:1 and 1:2 respectively by fusion using solid lipids: Precirol® ATO5 and beeswax, and the liquid lipid, Kolliphor® ELP. Briefly, the solid lipids were weighed using an analytical weighing balance (Ohaus Corp, USA) and melted together at 90 °C in a thermo-regulated water bath (HHW, China). The liquid lipid (oil) was incorporated while stirring with a glass rod until a homogenous, clear microemulsion was obtained. The homogenous mixture was stirred at room temperature (25 ± 2 °C) until solidification to obtain smooth lipid matrices. For cimetidine-loaded NLC formulation, 15 g of each ternary lipid system was melted at 90 °C in a thermostated water bath (HHW, China), and an appropriate amount of cimetidine was incorporated into the molten lipid. Sorbitol (4 % w/w) and Kolliphor® P188 (2 % w/w) were dispersed in distilled water to form the aqueous phase, and brought to the same temperature with the lipid melt. The aqueous phase was added to the molten lipid matrix with stirring using a magnetic stirrer (Jenway, Bibby Scientific, Essex, UK) for about 5 min at 500 rpm. Then, it was homogenized at 10,000 rpm (JB 90-S homogenizer, China) for 15 min to obtain a hot oil-in-water nanoemulsion which was lyophilized using a freeze-dryer (Yorco, York Scientific Ind., Pvt, India) to obtain water-free NLC. Six batches of NLC were formulated including drug-free batches. The water-free samples were stored in air-tight containers and refrigerated at 5 °C until needed [12].

**Physicochemical Characterization of NLC**

**Particle Size, Electric Charge, and Size Distribution**

The particle diameter, zeta potential and polydispersity index (PDI) of both cimetidine-loaded and unloaded NLC were measured by photon correlation spectroscopy (PCS) using a Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK) equipped with a green laser and employing a backscattering angle of 173 ° at 25 ± 2 °C. All the samples were dispersed in distilled water to obtain a suitable scattering intensity, and measurements were done in triplicate [14].

**Differential Scanning Calorimetry (DSC)**

The degree of crystallinity and polymorphism of cimetidine-loaded NLC formulations were studied by weighing out sufficient amounts of NLC containing approximately 5 mg of cimetidine into an aluminum pan, hermetically sealed and the thermal behaviour determined in the range of 60 – 280 °C under a 20 ml/min constant nitrogen flux at a heating rate of 10 °C/min using DSC (204 F1 Phoenix, Netzsch, Germany). Baselines were determined using an empty crucible, and all the thermograms were baseline-corrected, and data generated were analyzed using Proteus® Software for Windows [13].

**Table 1:** Composition of Nanostructured Lipid Carriers (NLC) of Cimetidine

<table>
<thead>
<tr>
<th>Batch</th>
<th>Kolliphor® P188 (%w/w)</th>
<th>Sorbitol (%w/w)</th>
<th>Cimetidine (% w/w)</th>
<th>Lipid matrix (%w/w)</th>
<th>Distilled water (%w/w), q. s</th>
</tr>
</thead>
<tbody>
<tr>
<td>A₁</td>
<td>2.0</td>
<td>4.0</td>
<td>0.1</td>
<td>15.0</td>
<td>100.0</td>
</tr>
<tr>
<td>A₂</td>
<td>2.0</td>
<td>4.0</td>
<td>0.3</td>
<td>15.0</td>
<td>100.0</td>
</tr>
<tr>
<td>A₃</td>
<td>2.0</td>
<td>4.0</td>
<td>-</td>
<td>15.0</td>
<td>100.0</td>
</tr>
<tr>
<td>B₁</td>
<td>2.0</td>
<td>4.0</td>
<td>0.1</td>
<td>15.0</td>
<td>100.0</td>
</tr>
<tr>
<td>B₂</td>
<td>2.0</td>
<td>4.0</td>
<td>0.3</td>
<td>15.0</td>
<td>100.0</td>
</tr>
<tr>
<td>B₃</td>
<td>2.0</td>
<td>4.0</td>
<td>-</td>
<td>15.0</td>
<td>100.0</td>
</tr>
</tbody>
</table>

* indicates ‘unloaded NLC for A₁ and B₁’, while A₂, A₃, B₁, and B₂ were loaded with cimetidine, q.s. indicates sufficient amount of distilled water used to make up the formulation.
Entrapment Efficiency (EE)

Different standard concentrations of cimetidine in 0.1N HCl were prepared and analyzed at a pre-determined wavelength of 215 nm using a UV spectrophotometer (Jenway 6505, Bibby Scientific, Essex, UK) to obtain the Beer-Lambert’s plot. Thereafter, 20 mg of each batch of cimetidine-loaded NLC was dispersed in 100 ml of 0.1N HCl, shaken vigorously in a 100 ml volumetric flask and centrifuged (800B-Electronic Centrifuge, China) continuously for 25 min at 5,000 rpm. The clear supernatant was carefully collected, while the residue was further washed with 0.1N HCl and filtered using Whatman filter paper (Ø = 90 mm, 11 µm). The supernatant and the filtrate were combined and adequately analyzed for actual drug content spectrophotometrically (Jenway 6505, Bibby Scientific, Essex, UK) at 215 nm in triplicates. The amount of drug encapsulated in each NLC was calculated with reference to the standard Beer’s plot for cimetidine. This study was repeated after 60 and 90 days to ascertain the level of uniformity of drug content of the loaded formulations. Then, EE was calculated using the equations below [10].

\[
EE(\%) = \frac{\text{Real cimetidine content}}{\text{Theoretical drug content}} \times 100 \quad \text{Eq. (1)}
\]

Stability Study

Stability study was carried out using dispersions of NLC from each batch stored at room temperature (27 ± 2 °C). Stability was determined by calculating EE as previously described, and measuring the pH of the dispersions in triplicate using a pH meter (Jenway 3505, USA). The pH meter was first calibrated with buffer solutions (pH 4 and 9) before the pH of the different batches was determined. Measurements were done after 60 and 90 days [10].

In Vitro Release Study

The dissolution medium consisted 900 ml of freshly prepared 0.1N HCl maintained at 37 ± 2 °C. An amount of NLC from each batch equivalent to 10 mg of cimetidine was placed in a dialysis membrane (MWCO = 8,000) (Spectrapor®, USA) already wetted, and containing about 2 ml of the dissolution medium. It was securely tied with a thermo-resistant thread to a retort stand while suspending the membrane in a 1000-ml beaker containing the dissolution medium. The beaker was mounted on a magnetic stirrer (Jenway, Bibby Scientific, Essex, UK) and agitation was provided by a magnetic stirring bar at 100 rpm for 12 h. At each pre-determined time interval starting from 10 min, 5 ml portion of the dissolution medium was withdrawn, appropriately diluted using fresh 0.1N HCl, and analyzed for drug content spectrophotometrically (UV spectrophotometer, Jenway 6505, Bibby Scientific, Essex, UK) at 215 nm in triplicates. Sink conditions were maintained by replacing the withdrawn medium with 5 ml of fresh 0.1N HCl immediately after each withdrawal. The experiment was also repeated using a market brand of normal release cimetidine tablet (Tagamet®, GlaxoSmithKline, USA). The amount of drug released at each time interval was determined with reference to the standard Beer’s plot for cimetidine in 0.1N HCl. The data obtained from the in vitro release study were used for kinetic modeling. Model fitting into zero order, first order, Higuchi, and Korsmeyer Peppas was done to study the mechanism of release of the NLCs [11].

Gastroprotective Pharmacodynamic Evaluation

Animal Care and Use Protocols

White albino rats (Wistar strain) of both sexes weighing 150 – 196 g were procured from the animal breeding centre, Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Nigeria. Animals were maintained and treated according to National Institute of Health (NIH) guidelines for animal care. All legal and ethical animal use protocols were approved by the Animal Care and Use Committee of the Faculty in compliance with the EU Directive 2010/63/EU for animal experiments. The animals were housed in rat cages, fed standard rodent diet (Vital Feeds Ltd., Nigeria) and allowed free access to clean, fresh water in glass water bottles ad libitum. They were acclimatized for 1 week prior to study, and a 12 h day/night cycle was maintained. Cage-side clinical observations of the rats were made throughout the study period. Previously reported methods were adapted for this evaluation with slight modifications [19].

Pyloric Ligation-Induced Gastric Ulceration

After seven days of habituation, forty-two (42) rats which were randomly selected, fasted for 24 h and grouped into seven (7) of six (6) rats each for this study. All the study rats received drug administration orally by gavage. Group I rats were administered 400 mg/kg body weight of the reference cimetidine brand (Tagamet®). Groups II, III, IV and V were administered 20 mg/kg body weight of cimetidine-loaded NLC
respectively. Group VI received 50 mg/kg body weight of solution of cimetidine powder as the positive control, while Group VII were administered 10 ml/kg body weight distilled water and served as the negative control. After 1 h, 20 mg/kg indomethacin was administered to all groups of rats as an ulcerative agent, and the rats were allowed free movement in their cages for 12 h to allow for the development of ulcer. After 12 h, the rats were anaesthetized in a chloroform chamber and their abdomens were surgically opened followed by ligation of their pylorus without obstructing blood supply. The abdomen was sutured, and the rats were allowed to stabilize without free access to drinking water. Finally, the rats were humanely euthanized in the chloroform chamber and their stomach was carefully isolated for further evaluation. The cardiac locus of the stomach was carefully dissected and its gastric contents were collected, and centrifuged at 4,000 rpm for 10 min. The volume of the clear supernatant was measured, and its hydrogen ion concentration (pH) determined using a pH meter (Jenway 3505, USA) [20].

Measurement of Ulcer Index

The stomach of the rats was rinsed with normal saline to remove blood clots, fixed in 10% formaldehyde and dehydrated in ascending grades of ethanol. Thereafter, the stomach was examined using a magnifying lens (MPS-30, Leica Microsystems, Wetzlar, Hesse, Germany) at ×100 magnifications to assess ulcer formation. The number of ulcer was defined using the following scale: normal coloured stomach = 0.0; red colouration = 0.50; spot ulcer = 1.00, haemorrhagic streak = 1.50; deep ulcer = 2.00; perforation = 3.00 [20]. The mean score for each rat was expressed as the ulcer index calculated using the formula:

\[
\text{Ulcer index (UI)} = U_n + U_k + U_p \times 10^{-1} \quad \text{Eq. (2)}
\]

Where \( U_n \) is the average number of ulcers per rat; \( U_k \) is the average number of severity score, and \( U_p \) is the percentage of rats with ulcers. Furthermore, the inhibition percentage of ulceration was estimated using the formula [20]:

\[
\text{Inhibition of ulceration (I %)} = \left( \frac{\text{Ulcer index}_{\text{control}} - \text{Ulcer index}_{\text{treated}}}{\text{Ulcer index}_{\text{control}}} \right) \times 100 \quad \text{Eq. (3)}
\]

Acetic Acid-Induced Gastric Ulceration

The same number of rats and grouping used for pyloric ligation-induced ulceration was adapted for this study. The rats were fasted for 24 h but with free access to water ad libitum. Drug administration was by oral gavage. Similar doses of the formulations and the control drugs were administered to the rats as described in the pyloric ligation method. After 1 h of intragastric treatment of rats, the animals were anaesthetized in a chloroform chamber, and a soft catheter was inserted into the colon of the rats via the rectum up to a depth of 8 cm and 2 ml (4%) dilute acetic acid solution was administered into the colon. The rats were maintained in a head down position for 5 min to prevent acetic acid solution leakage. After 12 h of ulcer induction, the animals were humanely euthanized in the chloroform chamber and the stomach was removed and ulceration scored according to the protocols already described above [21].

Statistical Analysis

Data from each individual experiment are expressed as the mean ± standard deviation (SD) of at least triplicate determinations. All statistical analysis were performed by one-way analysis of variance (ANOVA) using GraphPad Prism version 5.0 for Windows Software followed by Dunnnett’s post hoc least significant difference (LSD) test or Students’ t-test. Comparing with control group, differences were considered to be statistically significant at \( p < 0.05 \).

RESULTS

Selection of Lipid Excipients

The thermal data of the selected bulk, binary and ternary lipids are shown in Table 2. From the table, it could be seen that all the binary and ternary matrices produced endothermic peaks lower than the bulk starting lipids e.g. PATO5, BW and S154 had single endothermic melting peaks at 69.2, 64.0, and 59.0 °C with enthalpies of -24.31, -15.86, -8.87 mW/mg respectively. However, when the bulk lipids were mixed with KELP, their thermal properties showed steady decreases in endothermic melting points at 62.5, 62.7 and 53.2 °C with enthalpies of -15.72, -11.73, and -7.45 mW/mg respectively. Conversely, the ternary lipid matrix systems recorded lower endothermic melting peaks and enthalpies. PATO5/BW/KELP mixture had a melting peak of 50.2 °C, with an enthalpy of -3.47 mW/mg, PATO5/S154/KELP mixture showed a melting point of 53.7 °C with enthalpy of -5.27 mW/mg, while S154/BW/KELP blend recorded an endothermic melting peak of 55.2 °C and an enthalpy of -9.51 mW/mg. The ternary systems, PATO5/BW/KELP and
PATO5/S154/KELP were selected for further evaluation due to their low enthalpies.

**Drug Solubility and Crystallinity Study**

Visual observation showed the absence of cimetidine crystals in PATO5/BW/KELP matrix system during melting, mixing and cooling, but few drug crystals were observed in PATO5/S154/KELP matrix structure upon cooling at room temperature. Also, the solubilization of cimetidine in the oil and surfactant (KP188) dispersion alone was complete as no drug crystals were observed. Thermal data of the crystallization study is shown in Table 3. The table showed that cimetidine melted at 203.8 °C with an enthalpy of -6.80 mW/mg; while with 0.5 g cimetidine, it recorded a melting peak of 71.70 °C with an enthalpy of -5.44 mW/mg. Similarly, PATO5/S154/KELP matrix with 0.1 g of cimetidine melted at 74.3 °C with an enthalpy of -7.74 mW/mg, while with 0.5 g cimetidine, it melted at 84.75 °C and an enthalpy of -13.0 mW/mg. Finally, PATO5/BW/KELP was selected for further use since it encapsulated cimetidine at lower melting peaks and enthalpies with better solubility profile.

**Scanning Electron Microscopy (SEM)**

The particle morphologies (Figures 1a and b) and fibremetrics (Figures 2a and b) of cimetidine-loaded and drug-free ternary lipid matrix of choice were evaluated using SEM. The micrographs of the unloaded lipid matrix showed some deep, dark hollows

### Table 2: Composition and Thermal Profiles of Starting Lipid Matrices

<table>
<thead>
<tr>
<th>Lipid excipients</th>
<th>Peak melting point (°C)</th>
<th>Onset melting point (°C)</th>
<th>Enthalpy (mW/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bulk lipids</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precirol® ATO5</td>
<td>69.2</td>
<td>-</td>
<td>-24.31</td>
</tr>
<tr>
<td>Beeswax (white)</td>
<td>64.0</td>
<td>-</td>
<td>-15.86</td>
</tr>
<tr>
<td>Softisan® 154</td>
<td>59.0</td>
<td>-</td>
<td>-8.87</td>
</tr>
<tr>
<td><strong>Binary lipid blend (solid and liquid)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PATO5 – KELP (1:1)</td>
<td>62.5</td>
<td>50.3</td>
<td>-15.72</td>
</tr>
<tr>
<td>BW – KELP (2:1)</td>
<td>62.7</td>
<td>47.5</td>
<td>-11.73</td>
</tr>
<tr>
<td>S154 – KELP (1:2)</td>
<td>53.2</td>
<td>46.0</td>
<td>-7.45</td>
</tr>
<tr>
<td><strong>Binary lipid blend (solid and solid)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PATO5 – BW (1:1)</td>
<td>60.3</td>
<td>51.9</td>
<td>-18.85</td>
</tr>
<tr>
<td>PATO5 – S154 (2:1)</td>
<td>61.0</td>
<td>58.2</td>
<td>-15.01</td>
</tr>
<tr>
<td>S154 – BW (1:2)</td>
<td>62.1</td>
<td>60.7</td>
<td>-21.32</td>
</tr>
<tr>
<td><strong>Ternary lipid blend (solid and liquid)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PATO5 – BW – KELP (1:1)</td>
<td>50.2</td>
<td>45.8</td>
<td>-3.47</td>
</tr>
<tr>
<td>PATO5 – S154 – KELP (2:1)</td>
<td>53.7</td>
<td>49.1</td>
<td>-5.27</td>
</tr>
<tr>
<td>S154 – BW – KELP (1:2)</td>
<td>55.2</td>
<td>47.7</td>
<td>-9.51</td>
</tr>
</tbody>
</table>

Key: PATO5 – Precirol® ATO 5, BW – Beeswax, S154 – Softisan® 154, KELP – Kolliphor® ELP.

### Table 3: Drug Solubility and Crystallinity Study

<table>
<thead>
<tr>
<th>Ternary lipid matrix</th>
<th>Cimetidine (g)</th>
<th>Peak melting point (°C)</th>
<th>Enthalpy (mW/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precirol®ATO5-Beeswax-Kolliphor® ELP (1:1)</td>
<td>0.1</td>
<td>68.75</td>
<td>-3.47</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>71.70</td>
<td>-5.44</td>
</tr>
<tr>
<td>Precirol®ATO5-Softisan®154-Kolliphor® ELP (2:1)</td>
<td>0.1</td>
<td>74.30</td>
<td>-7.74</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>84.75</td>
<td>-13.00</td>
</tr>
<tr>
<td><strong>Drug</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cimetidine</td>
<td></td>
<td>203.80</td>
<td>-6.80</td>
</tr>
</tbody>
</table>
or pores for drug localization. In the cimetidine-loaded ternary matrix, there were no visible pores present but there were spherical particles spread throughout the surface of the matrix. The fibremetric analysis revealed an empty micrograph without fibres in the unloaded matrix. In contrast, the fibremetric analysis of the cimetidine-loaded lipid matrix was depicted by the presence of fibres all over the micrograph.

**Particle Size, Electric Charge and Size Distribution**

The result of the mean particle size, electric charge and particle size distribution of the cimetidine-loaded and unloaded NLC are presented in Table 4. The result showed that the formulations have particle sizes in the nanometer scale ranging from 113 – 153 nm, size distribution range of 0.13 – 0.41, an electric charge range of -31.5 to -37.1 mV. Batch B NLC (containing 1:2 PATO5/BW/KELP) gave the smallest particle size range of 113 – 130 nm, while batch A NLC (containing 1:1 PATO5/BW/KELP) gave the highest particle size range of 142 – 153 nm.

**Differential Scanning Calorimetry (DSC)**

The parameters of the obtained thermograms are summarized in Figures 3 and 4. Upon heating, the formulations produced clear transitions without shoulders and melting minima, and all the thermal properties were independent of the concentration of cimetidine. Representative batch A NLC formulation produced single endothermic melting peak at 90 °C with an enthalpy of -9.2 mW/mg, while representative
batch B NLC formulation recorded single endothermic melting peak at 88 °C with an enthalpy of -7.5 mW/mg.

Entrapment Efficiency (EE)

The result of EE of cimetidine-loaded NLC is presented in Table 5. From the table, batch A NLCs showed lower EE ranging from 85 – 91 % compared to the batch B formulations which recorded higher EE ranging from 88 – 96 %.

Stability Study

The results of the stability study are presented in Figures 5a and b for pH studies and in Table 5 for EE measurements. The pH of the NLCs was evaluated to ensure that encapsulation of cimetidine in NLC does not affect its stability in the presence of gastric acid. Generally, the pH of the formulations remained stable within the acidic scale of 3 – 4 across all the batches. For the measured EE, there was no remarkable changes (p<0.05) before and during storage throughout the study period.

In Vitro Release Study

The result of in vitro release study is shown in Figures 6a and b. Formulations containing 0.1 %w/w cimetidine (A₁ and B₁) showed somewhat burst release

<table>
<thead>
<tr>
<th>Batch</th>
<th>TDC (%w/w)</th>
<th>EE (%) (after 7 days)</th>
<th>EE (%) (after 60 days)</th>
<th>EE (%) (after 90 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A₁</td>
<td>0.1</td>
<td>85.0</td>
<td>83.7</td>
<td>80.1</td>
</tr>
<tr>
<td>A₂</td>
<td>0.3</td>
<td>91.4</td>
<td>89.5</td>
<td>85.0</td>
</tr>
<tr>
<td>B₁</td>
<td>0.1</td>
<td>88.6</td>
<td>84.2</td>
<td>81.9</td>
</tr>
<tr>
<td>B₂</td>
<td>0.3</td>
<td>96.9</td>
<td>93.8</td>
<td>90.3</td>
</tr>
</tbody>
</table>

TDC = Theoretical drug content; EE = Entrapment efficiency.
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of drug which was sustained gradually to a maximum release of 75 and 87 % after 11 h for A₁ and B₁ respectively. The reference commercial brand of cimetidine (Tagamet®) also showed a rapid release giving a maximum release of 40 % after 4 h, followed by reduced drug release. Formulations containing 0.3 %w/w of drug (A₂ and B₂) showed initial rapid release of drug followed by a gradual and sustained release of the drug to a maximum of 90 and 81 % after 11 h respectively for B₂ and A₂. The reference drug sample showed a similar release pattern as earlier observed. The kinetic study of drug release showed that all batches of the cimetidine-loaded NLCs (A₁, A₂, B₁, and B₂) followed the Higuchi square root of time model of release. Analysis of the correlation coefficient (r²) showed that the r² of the Higuchi model was higher (0.992 – 0.998) than the r² of zero order (0.662 – 0.987) and first order (0.778 – 0.971) models respectively. Also, drug release mechanism applying Korsmeyer-Peppas principle showed that the release exponent, 'n' values were within the range of 0.625 – 0.745.

Gastroprotective Pharmacodynamic Evaluation

Pyloric Ligation-Induced Gastric Ulcer

Table 6 shows the result of the effect of cimetidine-loaded NLC on pyloric ligation-induced gastric ulcer. From the result, it could be seen that all the batches of cimetidine-loaded NLC produced remarkable (p<0.05) anti-ulcer effect at a low dose (20 mg/kg) compared to the standard brand of cimetidine, Tagamet® administered at a high dose (400 mg/kg). In batch A, formulations A₁ and A₂ produced ulcer inhibition
between 80 – 90 %, decreased acidity with pH ranging between 4.5 – 4.8, comparable ulcer indices (6.74 and 6.71), and decreased gastric volume in comparison (p<0.05) with the negative control group. Similarly, for batch B, ulcer inhibition range of 81 – 88 % was produced by formulations B\textsubscript{1} and B\textsubscript{2}, decreased acidity with pH of 5, and gastric volume of ~6.5 ml in comparison (p<0.05) with the negative control group.

**Acetic Acid-Induced Gastric Ulceration**

Table 7 shows the result of the effect of cimetidine-loaded NLC on acetic acid-induced gastric ulceration. Precisely, formulations A\textsubscript{1} and A\textsubscript{2} of batch A gave ulcer inhibition between 80 – 82 %, decreased acidity with pH of 3.5, and decreased gastric volume compared (p<0.05) with the negative control (distilled water). Similarly, batch B formulations B\textsubscript{1} and B\textsubscript{2} produced ulcer inhibition ranging from 75 – 85 %, reduced acidity with pH between 4.1 – 4.5, and decreased gastric volume when compared with the negative control.

**DISCUSSION**

The decreased endothermic melting peaks obtained by mixing the bulk lipids with KELP indicates that the heterolipids influenced one another due to their heterogenous orientations, suggesting the creation of imperfections necessary for increased drug entrapment [10, 12, 22]. This argument subsists due to the corresponding lower enthalpies (-15.72, -11.73, and -7.45 mW/mg) generally recorded for the solid-liquid lipid binary matrices compared to the higher enthalpies (-24.31, -15.86, -8.87 mW/mg) recorded for the bulk lipids. Since lower melting enthalpies entail lower crystallinity, it means that the massive drop in enthalpies was as a result of the incorporation of KELP in the heterogenous binary matrix systems [11]. It is clear from these thermal profiles that solid-liquid lipid mixtures showed better ability to alter the crystalline packing of the lipids with the promise of increased drug localization than bulk or solid-solid lipid blends. The single endothermic melting peak of cimetidine indicates high purity and the absence of unstable polymorphs in the drug. The slight increases in endothermic melting peaks recorded for both drug-loaded ternary matrices compared with the unloaded matrices could be due to the presence of cimetidine, and the melting peak was low at 0.1 %w/w drug loading, but high at 0.5 %w/w loading, suggesting that high drug concentration requires more energy for molecular solubilization in the lipid matrix [12]. The low melting points and enthalpies

**Table 7: Effect of Cimetidine-Loaded NLCs on Acetic Acid-Induced Ulcer**

<table>
<thead>
<tr>
<th>*Group/batch</th>
<th>Dose (mg/kg)</th>
<th>pH</th>
<th>Gastric volume (ml)</th>
<th>Ulcer index</th>
<th>Percentage ulcer inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative Control</td>
<td>10 ml/kg</td>
<td>1.5 ± 0.51</td>
<td>5.5 ± 0.37</td>
<td>8.5 ± 0.50</td>
<td>-</td>
</tr>
<tr>
<td>Tagamet\textsuperscript{a} (Reference)</td>
<td>400.0</td>
<td>5.5 ± 0.41</td>
<td>1.5 ± 0.41</td>
<td>6.01 ± 0.12</td>
<td>69.29</td>
</tr>
<tr>
<td>B\textsubscript{2}</td>
<td>20.0</td>
<td>4.5 ± 0.44</td>
<td>2.0 ± 0.40</td>
<td>5.51 ± 0.70</td>
<td>85.17</td>
</tr>
<tr>
<td>B\textsubscript{1}</td>
<td>20.0</td>
<td>4.1 ± 0.40</td>
<td>1.5 ± 0.41</td>
<td>5.09 ± 0.71</td>
<td>75.11</td>
</tr>
<tr>
<td>A\textsubscript{2}</td>
<td>20.0</td>
<td>3.5 ± 0.38</td>
<td>1.5 ± 0.41</td>
<td>5.83 ± 0.43</td>
<td>82.01</td>
</tr>
<tr>
<td>A\textsubscript{1}</td>
<td>20.0</td>
<td>3.5 ± 0.38</td>
<td>1.0 ± 0.25</td>
<td>5.17 ± 0.72</td>
<td>80.17</td>
</tr>
<tr>
<td>Cimetidine powder</td>
<td>50.0</td>
<td>4.5 ± 0.41</td>
<td>1.5 ± 0.41</td>
<td>6.76 ± 0.12</td>
<td>50.47</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Number (n) of rats per group = 6.
recorded indicate that despite the poor lipophilic nature of cimetidine, it attained high loading in the lipid matrices [23].

The deep, dark hollows or pores observed in the micrograph of unloaded lipid matrix indicate there was molecular rearrangement in the crystalline structures of the lipids creating imperfections for drug entrapment. The above finding is in agreement with earlier reports which suggested that the shape of solid lipid nanocarriers is influenced by the nature of lipids used in their formulation, and spherical particles are usually obtained from chemically polydispersed lipids [24, 25]. The absence of pores in the cimetidine-loaded matrix and the presence of spherical particles spread throughout the surface of the matrix perfectly confirm the occurrence of low crystal order in the lipids resulting in high drug entrapment. It should be noted that the micrograph presents three-dimensional photographs in a two-dimensional way, especially for a freeze-fractured sample because the matrix lipid does not recrystallize in the condition, and so particles may not appear spherical when viewed edge-on. Furthermore, fibremetric analysis is an important characteristic of particle engineering because it provides insight into the physical orientation of the particles as well as their stability. Phenom Prox SEM is equipped with the fibremetric software which allows the view and measurement of micro- and nano- fibres with unmatched accuracy revealing the distribution of particles within a sample. The presence of fibres in the micrograph indicates drug distribution in a drug-loaded matrix, while the absence of fibres suggests no drug distribution in an unloaded matrix. The reduction in the size of the microarea available for drug solubilization indicated that the homogenous distribution of cimetidine in the lipid matrix produced a rearrangement in the molecular orientation of the drug-loaded lipid matrices reducing the tendency of chance crystallization, leading to a decrease in the microarea occupied by the lipids [18].

It is important to note that the differences in particle sizes between batches A and B NLC were quite significant (p<0.05) as could be verified from their deviation values. Essentially, particle size and size distribution were not influenced by drug loading. This is in agreement with earlier reports [26, 27]. It has been suggested that when formulated in smaller sizes, nanoparticles produce prolonged biological activities due to their adhesive property [28]. This is particularly important for cimetidine with low membrane permeability because in the nanometer size range, it will have increased contact time and bioaccessibility with gastrointestinal tissues, improved dissolution rate and unhindered trafficking across cells and membranes [29]. The narrow size distribution entails that the nanoparticles would less likely experience coalescence and other physical instability, which would encourage particle size growth. Electrical charge of the NLC refers to the zeta potential, which is a vital indicator of the potential physical stability of the colloidal system, and high negative (above -30 mV) or high positive (above +30 mV) zeta potential is indicative of colloidal dispersion stability [30]. Considering the zeta potential, the high stability of batch B NLC could be due to the presence of high concentration of the liquid lipid which synergized with the solid lipids to create more spaces for complete drug solubilization, and the overall favourable interfacial behaviour between the lipids and the surfactant [31, 32].

It has been suggested that mixed lipids with low melting transitions and enthalpies are ideal for the formulation of NLC or SLN [33]. In comparison, the melting transition and enthalpy of the batch B formulation prepared with higher amount of the oil (KELP) were lower than that of batch A formulation which contain a lesser concentration of the liquid lipid. The lower enthalpy suggests that batch B formulations do not contain a highly ordered crystalline packing and would likely encapsulate a high amount of the payload (cimetidine) [34].

The result indicated that EE was influenced by drug loading, as formulations with high drug loading recorded the highest (p=0.05) percentage drug encapsulation, suggesting favourable interaction between cimetidine and the heterolipid carrier system. This corroborated the result from thermal analysis (DSC) which indicated that the drug carrier system was less crystalline with imperfections created for increased accommodation of guest drug molecules, and a low tendency for drug expulsion from the lipid core due to prominent differences in the packing structure of the formulation lipids, especially the solid lipids – Precirol ATO5 (C-16) and beeswax (C-30 -32) [35]. The result further showed that formulation B2 had the best drug entrapment efficiency (96.9 %) compared with A2 (91 %), though they were loaded with the same amount of drug. This could be due to the high liquid lipid content in batch B formulations which triggered extensive crystal lattice disorderliness favouring increased solubilization of cimetidine.

The acidic pH of the cimetidine NLC indicates that the fatty acid content of the lipid carriers might have
influenced the pH of the NLCs. Cimetidine is a weak base, and it is expected to show stability in an acidic environment. The minor variations in pH recorded for the formulations depicted by very narrow standard deviations, suggests that there was no significant (p<0.05) changes in the pH values recorded at the storage temperature [18]. The pH result is largely acceptable since it is very important for cimetidine to be stable in the hostile acidic environment of the GIT upon oral administration for the effective treatment of PUD. Furthermore, gastric stability is essential, especially when the carrier system docks in the small intestine, and is digested by intestinal enzymes yielding mixed micelles typical of lipids resulting in the improved bioavailability of cimetidine [36]. In addition, the stability of the formulations might also be due to the stabilization effect of the surfactant used [37], or due to the bioadhesive nature of the nanoparticles with possible sustained release property [38]. The non-significant variation in the measured entrapment efficiency of the cimetidine-loaded NLC confirms that the drug was properly accommodated and entrapped within the crystal lattice of the lipid nanocarrier [39].

From the result of in vitro release study, the rate of drug release depends on the concentration of the loaded drug, and the amount of cimetidine released from the NLC batches increased significantly (p<0.05) with increased drug loading [39]. The maximum drug release of 40 % from the commercial brand of cimetidine (Tagamet®) after 4 h confirms the clinical pharmacokinetic profile of normal release Tagamet® tablets administered orally which produces maximum inhibition of gastric acid secretion after 4 h. Taken together, batches A₁ and B₁ produced remarkably significant (p<0.05) and better release of cimetidine than Tagamet®. The reference drug sample showed a similar release pattern as earlier observed. The burst release could be due to unentrapped cimetidine adsorbed at the surface of the NLCs following solubilization in the surfactant micelles; however, it might ensure a faster onset of action [40]. Furthermore, the biphasic (burst and sustained) release patterns observed is in contrast with some reports [15, 37], but is in agreement with other reports [41]. The increased drug release recorded for the cimetidine-loaded NLCs confirms the high entrapment efficiency earlier observed for the lipid nanocarriers, and it could be attributed to the physical state of the lipids as increased liquid lipid content resulted in improved drug entrapment and release [42]. It is also suspected that the small size of the lipid particles could be responsible for the increased release of cimetidine, since it has been suggested that possession of small particulate size is an essential condition for improved drug release [43].

The Higuchi model of release followed by the formulations indicates that the NLCs had diffusion-controlled release of cimetidine from a heterogenous matrix system, which is characteristic of NLCs. Since the ‘n’ values are greater than 0.43 but less than 1.00, it suggests that the mechanism of release of cimetidine from the NLCs was non-Fickian (anomalous) diffusion. This means that drug release from the NLCs was controlled mainly by diffusion and erosion of the matrix system of the samples [11].

The result obtained from pyloric ligation-induced gastric ulcer study conforms to literature [20]. The NSAID, indomethacin, has been shown to induce gastric ulcer through inhibition of prostaglandin synthesis, increased expression of interleukin-1 (IL-1), generation of reactive oxygen species (ROS) and induction of apoptosis, resulting in amongst other defects glutathione depletion [44]. Therefore, the result could be a significant indication that the cimetidine-loaded NLC attenuated indomethacin-induced acid production mechanisms and related aggressors in the GIT, while potentiating the effect of the gastric endogenous defense mechanisms [45]. Within a batch, it is clear from the result that ulcer inhibition was dose-dependent as formulations loaded with 0.3 %w/w cimetidine produced the highest inhibition of gastric acid production compared with those loaded with 0.1 %w/w cimetidine. This confirms the observation made in the in vitro release study. As expected, the negative control group which was administered distilled water did not inhibit ulceration, while the commercial brand, Tagamet®, produced the highest inhibition of ulcer probably due to the high dose used in the study compared to the low dose of the nanoparticles. It could be seen that the nanoformulations produced better percentage ulcer inhibition than dispersions of pure cimetidine, and this suggests that cimetidine could be better absorbed from NLC than in the salt form. Furthermore, the very small size of the nanoparticles could have favoured high drug absorption resulting in significant (p<0.05) ulcer inhibition, since it has been shown that particle size is an important drug carrier parameter which influences drug release, cellular and tissue trafficking and bioavailability [46].

The acetic acid-induced gastric ulceration investigated the gastroprotective activity of cimetidine-
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loaded NLC using acetic acid-induced gastric ulceration method, which is similar to chronic gastric ulcers in human because it presents the same clinicopathological conditions, and affects the entire length of the gastrointestinal tract (GIT) due to its overwhelming effects on the mucosal defenses [21]. It could be observed from the result obtained that the formulations produced very significant (p<0.05) anti-ulcer activity compared to the reference (Tagamet®) drug sample despite the high dose of the reference brand administered to the rats. This is similar to the scenario observed with the activity of the cimetidine-loaded NLC in pyloric ligation-induced gastric ulcer. The result suggests that the cimetidine-loaded NLC regulated acid related necrotic effects of acetic acid and other noxious factors in the gastrointestinal tract of the rats with enhanced activity of the endogenous protective factors, such that mucosal injury does not arise or is ameliorated. Since acetic acid induces gastric ulcer by decreasing gastric blood flow, solubilizing mucus constituents in the stomach with elevated influx of Na⁺ and K⁺ ions followed by loss of H⁺ ions resulting in increased acid secretion into the mucosa [47] which overwhelm the mucosal defenses, it could be submitted that the nanoformulations inhibited ulcer formation with restoration of the defense factors following antagonistic effects on the acetic acid ulcer induction mechanisms and the antisecretory activity of cimetidine [48].

CONCLUSION

Cimetidine NLC was formulated using ternary blend of heterogeneous lipids with heterogeneous molecular lattice structures which favoured drug encapsulation. The formulations had desirable nanosize, electric surface charge and thermal properties with low crystallinity. In vitro release study showed dose-dependent sustained release of cimetidine from the NLCs, followed Higuchi kinetic model with non-Fickian diffusion mechanism of drug release. Formulation as NLC did not inhibit the antisecretory activity of cimetidine, and it is possible that the small size of the particles enhanced mucosal and tissue permeation for improved gastroprotective effect and ulcer healing. Overall, it could be concluded that NLC is a valuable drug carrier system for cimetidine for improved gastroprotective activity

ACKNOWLEDGEMENT

The authors thank Cera Alba Pellets, Hilden, Germany for the kind gift of white beeswax. They also appreciate Gattefosssé, Saint-Priest Cedex, France for the gift of Precirol®ATO5 (PATO5). The authors thank Fa.Condea Chemie GmbH, D-58453Witten, Germany for the generous donation of Softisan®154. We also thank BASF SE, Ludwigshafen, Germany for the kind provision of Kolliphor®ELP (KELP) and Kolliphor®P188 (KP188).

REFERENCES


[42] Aditya NP, Chimote G, Gunalan K, Banerjee R, Patankar S, Madhusudhan B. Curcuminoids-loaded liposomes in...
https://doi.org/10.1016/j.exppara.2012.04.010

https://doi.org/10.1016/j.ijpharm.2008.06.002


https://doi.org/10.1074/jbc.M413398200

https://doi.org/10.1016/j.jep.2005.08.064

https://doi.org/10.3748/wjg.v11.i45.7203

https://doi.org/10.1016/S1043-6618(03)00155-5