FORMULATION AND CHARACTERIZATION OF AN INTRAGASTRIC FLOWING DRUG DELIVERY SYSTEM OF DOXORUBICIN HYDROCHLORIDE: IN VITRO-IN VIVO RELEASE STUDY

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ABSTRACT

The objective of the present study was to develop floating microspheres of doxorubicin in order to achieve an extended retention in upper gastrointestinal tract, which may result in enhanced absorption and thereby improved bioavailability. Microspheres were prepared by emulsification extraction technique using pectin as polymer and casein as emulsifier. The 3rd full factorial design was employed to evaluate contribution of independent variables, pectin-casein ratio (X1) and stirring speed (X2) on dependent variables, % buoyancy and % drug release. The optimum batch was F6 which showed particle size 68.8 µm, 78% entrapment efficiency and 8% drug release after 8 hrs. Doxorubicin microspheres exhibited significant improvement in bioavailability and drug release compared to pure drug. In vitro-in vivo study of F6 formulation indicated that the microsphere remained buoyant for to having lower density than the fluids. They are not subjected to 'all or nothing' gastric emptying nature of single unit system. It releases the drug in controlled fashion. The pH of the stomach can be increased either by frequent administration of water or administration of alkali rich meal or by antacid. The present investigation describes the formulation development of an intragastric floating drug delivery system of doxorubicin hydrochloride and in vitro-in vivo release study at higher gastric pH.

Keywords: Porous microspheres, Doxorubicin, Emulsification extraction method.

INTRODUCTION

Oral drug delivery of drugs is by far the most preferable route of drug delivery due to ease of administration, patient compliance and flexibility in formulation etc. Oral sustained drug delivery formulations show some limitations connected with the gastric emptying time variables and too rapid gastrointestinal transit could result in incomplete drug release from the device into the absorption window leading to diminished efficacy of the administered dose. To overcome this problem several attempts have been made to develop oral dosage forms capable of having prolonged retention in the stomach to extend the duration of drug delivery. It is evident from the recent research and patent literature that interest in novel dosage forms is unexpectedly increasing. Example of such systems are gastro retentive floating dosage forms, these dosage forms are based on different mechanism which include floatation, mucoadhesion, sedimentation, expansion, modified shape system or by simultaneous administration of pharmacologic agents that delay gastric emptying. Drug candidate suitable for this system are 1) which have site specific absorption in the stomach, or upper part of intestine, 2) drugs required to exert local action therapeutic action in stomach 3) drugs unstable in the lower part of gastrointestinal tract 4) drugs insoluble in intestinal fluids 5) drugs with variable bioavailability.

Doxorubicin (Dox), an anthracycline antibiotic and one of the most widely used anticancer agents, shows high antitumor activity. However, its therapeutic effects are limited due to its dose dependent cardiotoxicity and myelosuppression. Indeed, nearly 60% drugs with variable bioavailability are gastro retentive drugs, which have site specific absorption in the stomach, or upper part of the intestine 3) drugs unstable in the lower part of gastrointestinal tract 2) drugs required to exert local action therapeutic action in stomach 1) which have site specific absorption in the stomach, or upper part of intestine, 4) drugs insoluble in intestinal fluids 5) drugs with variable bioavailability.

Doxorubicin is less because it is eliminated by the first-pass extraction of the cytochrome P450-dependent metabolic process and the over expression of the multidrug efflux pump transporter P-glycoprotein (P-gp) which is rich in the intestine, liver and kidney, thus making it difficult to administer doxorubicin via oral route along with poor permeability. The general idea is to apply P-gp/P450 inhibitors such as cyclosporine A to suppress the elimination process. But these inhibitors suppress body's immune system and cause medical complications. Moreover, molecules like cyclosporine have their own side effects thus making it more difficult to incorporate them into drug delivery system along with anticancer agents. Advanced drug delivery strategies can offer alternatives which can circumvent the issues associated with drug's toxicity and on the other hand can lead to enhanced therapeutic performance by increasing the bioavailability of the drug.

Another reason of low bioavailability of doxorubicin is degradation of doxorubicin in the pH region 0.4-2.1; here deactivation of the amino sugar moiety occurs. Doxorubicin is stable at pH above than 3. Bioavailability of drug can be enhanced by increasing pH.

Therefore, porous microspheres (GRDFS) have emerged as an efficient means of prolonging gastric residence time, targeting stomach mucosa, and also improving the bioavailability. Microspheres remain buoyant for to having lower density than the gastric and intestinal fluids. They are not subjected to ‘all or nothing’ gastric emptying nature of single unit system. It releases the drug in controlled fashion. The pH of the stomach can be increased either by frequent administration of water or administration of alkali rich meal or by antacid. The present investigation describes the formulation development of an intragastric floating drug delivery system of doxorubicin hydrochloride and in vitro-in vivo release study at higher gastric pH.

MATERIALS

Doxorubicin was purchased from RCG Life Sciences Limited, Navi Mumbai. Casein purchased from Loba chemicals and Pectin from Southern Citrus Products Pvt. Ltd, Gudur, AP (India). All other chemicals were of analytical reagent grade.

METHODS

Analytical estimation of doxorubicin

Doxorubicin was estimated by UV-Vis spectrophotometric method (Shimadzu UV 1601, Kyoto, Japan). Solution of drug was prepared in distilled water; the absorbance was measured at 495 nm spectrophotometrically from 2.0 to 20.0 µg concentration.

Experimental design

A 3rd randomized full factorial design was adopted to optimize the variables. In the design two factors were evaluated, each at 3 levels, and experimental trials were performed at all nine possible combinations. In the present investigation, the ratio of polymer: emulsifier (X) and stirring speed (X) were selected as independent variables. The particle size, % drug entrapment, % Buoyancy and time required for 50% drug release were selected as dependent variables.
Preparation of microsphere

Porous microspheres were prepared by emulsification extraction method which was previously described by Shruiti et al. In brief, 15% w/v solution of casein and pectin in different ratio were taken in 10 ml deionized water (60°C) then added to 60 ml Soya oil preheated to same temperature. The dispersion was stirred to obtain emulsion and rapid cooling and 150ml previously cooled acetone was added to obtain solid microspheres that were filtered, washed with acetone and dried in dessicator and stored in well closed container.

Determination of particle size and its size distribution

The particle size of the microsphere was determined by using optical microscopy method. Approximately 500 particles were counted for particle size using a calibrated optical microscope. The shape and surface morphology of the microsphere was investigated using scanning electron microscopy (photograph no 1). Photomicrographs were observed at 50x magnification opened with an acceleration voltage of 10kV and working distance 9.1mm was maintained.

Morphological study of microsphere

The thermal behavior of the floating and nonfloating microspheres. These healthy albino mice were used to monitor the in vivo transit behavior of the floating and nonfloating microspheres. These mice were divided into 2 groups (group A and group B). The location of the formulation in the stomach was monitored by keeping the subjects in front of E-CAM gamma camera with SPECT technology.

In vitro dissolution studies

In Vitro dissolution studies were performed using US Pharmacopeia XXII dissolution apparatus II (paddle type). An accurate weighed sample (40 mg) of optimized porous microsphere was dropped into 900 ml of acetate buffer pH 4.0 maintained at a temperature at 37°C ± 0.5°C and stirred at a speed of 50 rpm. At different time intervals, a 10ml aliquot of the sample was withdrawn and the volume was replaced with an equivalent amount of plain dissolution medium kept at 37°C. The collected samples were filtered and analyzed at λmax 496nm using a UV-Visible spectrophotometer against acetate buffer pH 4.0 taken as blank. Percentage drug dissolved at different time intervals was calculated using Lambert-Beer’s equation. The drug release was calculated using various models. The average values of t50 for batches F1 to F9 are mentioned in Table 1. The percentage release of batches F6 is shown in Figure 4.

In vitro cytotoxicity analysis of doxorubicin loaded microsphere on kato iii

Human gastric cancer cell line

The KATO III human gastric cancer cell line were purchased from National Centre for Cell line Pune and cultured in Jawaharlal Nehru Cancer Research Centre and Hospital, Bhopal. MTT assay was performed, ([3 – (4, 5 – dimethylthiazol) 2] V). 2, 5 – diphenyl tetra sodium bromide] is a pale yellow substrate that is cleaved by living cells to yield a dark blue formazon product. This process requires active mitochondria and even freshly dead cells do not cleave significant amount of MTT. Thus the amount of MTT cleaved is directly proportional to the number of viable cells present, which is quantified by colorimetric methods. This assay was performed using the standard operating procedures. To examine the effects of pure Dox and Dox loaded porous microspheres, the cells were treated with 0.001, 0.01, 0.1, 1 and 10 mg/ml of Dox and similar concentrations of optimized microspheres (F6). Cell lines maintained in appropriate condition were seeded in 96 well plates is fed in 96 well plates and incubated at 37°C, 5% CO2 for 1-5 days, MTT reagent was added to the wells and incubated for 4 hours, the dark blue formazon product formed by the cells as dissolved DMSO under a safety cabinet and read at 550nm in Elisa reader. Percentage inhibitions were calculated and plotted with the concentrations used to calculate the IC50 values.
The gamma-studio study was performed as per the guidelines approved by the Ethical Committee of School of Pharmacy, Chouksey Engineering College (1275/ac/09 / CPCSEA/2010/07) and the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

**Antitumor activity**

The in vivo study was performed as per the guidelines approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. The Institutional Animals Ethical Committee of School of Pharmacy, Chouksey Engineering College approved the protocol for the study. Swiss albino male/female aged 8 to 9 weeks was used. The animals were kept under a standard 12/12 light/dark cycle and allowed to move and carry out normal activities and allowed to take water frequently until the formulation had emptied the stomach completely to increase the pH of stomach. The animals were freed and allowed to move and carry out normal activities and allowed to take water frequently until the formulation had emptied the stomach completely to increase the pH of stomach.

**RESULTS AND DISCUSSION**

Porous microspheres of doxorubicin were successfully prepared by emulsification extraction method. A statistical model incorporating interactive polynomial term was used to evaluate the response

\[ Y = b_0 + b_1 x_1 + b_2 x_2 + b_1 x_1 x_2 + b_2 x_1^2 + b_3 x_2^2 \]

Where, \( Y \) is the dependent variable, \( b_i \) is the estimated coefficient for the factor \( X_i \). The main effects \( (X_1 \) and \( X_2 \) represent the average results of changing one factor at a time from its low to high value. The interaction terms \( (X_1 X_2) \) show how the responses changes when two factors are simultaneously changed. The polynomial terms \( X_1^2 \) and \( X_2^2 \) are included to investigate nonlinearity.

### Table 1: 3² Full factorial design layout.

<table>
<thead>
<tr>
<th>Batch Code</th>
<th>Variable levels in coded form</th>
<th>Particle size analysis (µm)</th>
<th>Buoyancy (%)</th>
<th>Drug Entrapment (%)</th>
<th>t80% (Min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>-1</td>
<td>96.1±0.23</td>
<td>87.0±0.45</td>
<td>38.0±0.89</td>
<td>23.0±2.78</td>
</tr>
<tr>
<td>F2</td>
<td>-1</td>
<td>87.6±0.45</td>
<td>84.0±0.34</td>
<td>40.0±0.48</td>
<td>24.9±2.02</td>
</tr>
<tr>
<td>F3</td>
<td>-1</td>
<td>90.0±0.35</td>
<td>82.0±0.67</td>
<td>41.0±0.87</td>
<td>25.6±1.86</td>
</tr>
<tr>
<td>F4</td>
<td>0</td>
<td>88.9±0.26</td>
<td>78.0±0.65</td>
<td>69.0±0.88</td>
<td>39.0±2.48</td>
</tr>
<tr>
<td>F5</td>
<td>0</td>
<td>68.8±0.98</td>
<td>70.0±0.96</td>
<td>40.5±1.90</td>
<td>42.0±1.99</td>
</tr>
<tr>
<td>F6</td>
<td>0</td>
<td>83.0±0.23</td>
<td>76.0±1.23</td>
<td>74.0±0.93</td>
<td>39.5±1.23</td>
</tr>
<tr>
<td>F7</td>
<td>0</td>
<td>76.5±0.48</td>
<td>62.0±0.78</td>
<td>70.0±0.60</td>
<td>37.9±2.03</td>
</tr>
<tr>
<td>F8</td>
<td>1</td>
<td>76.0±0.98</td>
<td>68.0±1.50</td>
<td>74.0±0.88</td>
<td>37.6±0.98</td>
</tr>
<tr>
<td>F9</td>
<td>1</td>
<td>69.3±0.95</td>
<td>69.0±0.88</td>
<td>70.0±0.60</td>
<td>39.0±2.79</td>
</tr>
</tbody>
</table>

### Table 2: Angle of repose, carr’s index and hausner’s ratio as an indication of flow properties.

<table>
<thead>
<tr>
<th>Angle of repose (θ)</th>
<th>Carr’s index</th>
<th>Hausner’s ratio</th>
<th>Type of flow</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;20</td>
<td>12-16</td>
<td>&lt;1.25</td>
<td>Good</td>
</tr>
<tr>
<td>30-40</td>
<td>18-21</td>
<td>-</td>
<td>Fair to passable</td>
</tr>
<tr>
<td>-</td>
<td>25-35</td>
<td>&gt;1.25</td>
<td>Poor</td>
</tr>
<tr>
<td>-</td>
<td>33-38</td>
<td>1.25-1.5</td>
<td>Very poor</td>
</tr>
<tr>
<td>&gt;40</td>
<td>&gt;40</td>
<td>-</td>
<td>Extremely poor</td>
</tr>
</tbody>
</table>

### Table 3: Micromeritic properties.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Code</th>
<th>Mean Particle Size (µm)</th>
<th>Angle of Repose (θ)</th>
<th>Carr’s Index (%)</th>
<th>Hausner’s Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F1</td>
<td>96.1±0.34</td>
<td>29.4±0.44</td>
<td>15.1±0.51</td>
<td>1.16±0.74</td>
</tr>
<tr>
<td>2</td>
<td>F2</td>
<td>87.6±0.65</td>
<td>27.3±0.63</td>
<td>14.9±0.48</td>
<td>1.15±0.85</td>
</tr>
<tr>
<td>3</td>
<td>F3</td>
<td>80.0±0.33</td>
<td>25.9±0.84</td>
<td>13.6±0.76</td>
<td>1.13±0.97</td>
</tr>
<tr>
<td>4</td>
<td>F4</td>
<td>88.9±0.28</td>
<td>26.6±0.76</td>
<td>13.4±0.63</td>
<td>1.15±0.58</td>
</tr>
<tr>
<td>5</td>
<td>F5</td>
<td>74.9±0.96</td>
<td>22.3±0.58</td>
<td>12.4±0.38</td>
<td>1.14±0.49</td>
</tr>
<tr>
<td>6</td>
<td>F6</td>
<td>68.0±0.52</td>
<td>23.6±0.38</td>
<td>11.6±0.54</td>
<td>1.14±0.85</td>
</tr>
<tr>
<td>7</td>
<td>F7</td>
<td>88.4±0.26</td>
<td>26.8±0.91</td>
<td>13.5±0.66</td>
<td>1.66±0.43</td>
</tr>
<tr>
<td>8</td>
<td>F8</td>
<td>76.5±0.75</td>
<td>25.4±0.65</td>
<td>12.2±0.38</td>
<td>1.15±0.71</td>
</tr>
<tr>
<td>9</td>
<td>F9</td>
<td>69.3±0.24</td>
<td>24.2±0.39</td>
<td>11.8±0.64</td>
<td>1.14±0.22</td>
</tr>
</tbody>
</table>

* **n = 3, all values ± standard deviation, statistically significant at 0.05 level.**
The statistical analysis of the factorial design batches was performed by multiple regression analysis using Microsoft Excel. To demonstrate graphically the influence of each factor on response, the response surface plots were generated using Sigma Plot Software version 11.0 (Jandel Scientific Software, San Rafael, CA). The particle size, % drug entrapment, % Buoyancy, and time required for 80% drug release for the nine batches (F1 to F9) showed a wide variation 68.8 – 96.1µm, 38.0–78.0%, 58.0–87.0% and 230 -485 min respectively (Table no.1). The data clearly depicts that the Particle size, % drug entrapment, % Buoyancy and time required for 80% drug release values are strongly dependent on the selected independent variables. The polynomial equations can be used to draw conclusions after considering the magnitude of coefficient and the mathematical sign it carries.

Particle size

\[ y = +76.03 - 4.91 x_1 - 9.21 x_2 + 5.45 x_1^2 + 2.25 x_2^2 - 0.75 x_1 x_2 \]

% Drug entrapment

\[ y = +71.66 + 17.33 x_1 + 2.66 x_2 - 1.5 x_1^2 + 1.0 x_2^2 - 0.25 x_1 x_2 \]

% Buoyancy

\[ y = +74.33 - 10.5 x_1 - 3.83 x_2 - 1.5 x_1^2 + 0.5 x_2^2 - 0.75 x_1 x_2 \]

80% t

\[ y = + 402.44 + 9.83 x_1 + 9.83 x_2 - 87.16 x_1^2 + 3.83 x_2^2 - 5.75 x_1 x_2 \]

Three dimensional response surface plots for all response variables are presented in Figure 1(A), (B), (C) and (D) which are very useful to study the interaction effects of the factor (X₁X₂) on the responses.

The response surface depicts effect of factor contribution at different levels on studied response.

Figure 1(A) depicts a decline in particle size with increase in stirring speed. At higher emulsifier to polymer ratio, particle size is more. The lowest particle size recorded at medium level of emulsifier and polymer ratio. Figure 1(B) reveals increase in the value of % entrapment efficiency with increase in polymer content of the formulation, followed by an increase with decreasing value of emulsifier and again very much increase in % EE with lesser emulsifier content, the effect of emulsifier being significant.

Figure 1(C) reveals decrease in value of % buoyancy with increase in polymer content of the formulation. As the quantity of emulsifier is increased, it also increases the % buoyancy. Particle size slightly affects the buoyancy of formulation.

Figure 1(D) shows that a maximum of 80% drug release was obtained with medium level of emulsifier to polymer ratio. With increase in polymer content slight decrease in release was observed. High emulsifier content, results in a more significant decrease.

High emulsifier content, results in a more significant decrease. Figure 2 shows scanning electron microscopic photographs of microspheres which are spherical with rough surface.

Figure 3(A), (B) and (C) are the characteristic peaks of the pure drug, drug loaded microsphere and placebo microsphere. The drug remained unaltered in the FTIR spectra of doxorubicin microspheres. The peaks which are in pure drug spectra are also present in spectra of microsphere but doesnot exist in placebo microsphere. FTIR analysis reveals that there is no interaction between drug and drug loaded microsphere.
TG-DTA experiments were performed to investigate the physical state of the drug or polymer in the microspheres, because this aspect could influence the in vivo and in vitro release of the drug from the system. The specimen is continuously heated (or cooled) with a steady heating/cooling rate. Figure 4(A) illustrate a typical TG-DTA scan (heat flow vs. temperature), showing the melting of well-known drug doxorubicin at 223.25°C which is indicated by endothermic peak (heat absorption). The melting peak of the drug was totally disappeared in the thermogram of loaded microsphere evidencing the absence of crystalline drug in the microsphere sample. Loaded microspheres showed that exothermic melting transition started at 198.32°C (Figure 4(B)). The cross linking of polymer molecules is the exothermic process, resulting in a positive peak in the TG-DTA curve. Therefore, it could be concluded from the TG-DTA of optimized batch that doxorubicin in microspheres was in an amorphous or a solid solution state in the polymer matrix after the production. Size of microsphere greatly affects the flow properties. Particles or microspheres having a smaller size showed good flow properties.
Figure 4: TG-DTA thermogram scan of (A) pure doxorubicin (B) drug loaded microsphere

Figure 5: Percentage drug release data (a) without model fitted and (b) with model fitted.
Figure 6: IC₅₀ (A) pure drug (B) drug loaded microsphere.

Figure 7: Gamma scintillography images of group a and group b.
Microparticulate drug delivery systems are crucial where the sustained release of drug is desired for a longer time period and chronic illness like cancer forms no exception to this. One of the desired attributes of oral chemotherapy is reduced dosage frequency and accumulation of the dosed drug in the tumor tissues by enhanced permeation retention that can be attained using microparticles. In vitro release of doxorubicin mainly depends upon the polymer and emulsifier ratio. Figure 5(A) reveals that the drug release was sustained for more than 8 hrs and in controlled manner. To ascertain the drug release mechanism and release rate, data of formulation F6 were fitted by using PCP Diss V3 dissolution software. The model selected were zero order ($r=0.7578$, $k=13.0591$), first order ($r=0.9664$, $k=-0.2319$), Higuchi ($r=0.9905$, $k=30.5968$), korsemeyer peppas ($r=0.9915$, $k=31.8977$) and Hixon crowell ($r=0.9247$, $k=46.26$) as represented in figure 5(B). All models pass t test. The result suggests that for formulation F6 best fit model was found to be Korsemeyer Peppas model. The value of correlation coefficient was 0.9592. The value of slope and intercept were found to be 0.7714 and 10.1091. The $n = 0.4734$ value shows fickian release pattern from formulation F6.

The inhibitory potency of the pure drug on the Kato III cell line was compared by using the IC50 value. The IC50 represents the concentration of the drug at which 50% of inhibition is produced. Pure drug sample showed good drug release at pH 4.0. Drug loaded microspheres showed very significant results compared with the pure drug. Figure 6(A) and (B) for pure drug and drug loaded microspheres showed very significant results compared with the formulation F6 both showed cytotoxicity against Kato III cells. Hence the formulation can be effectively tested for its anticancer activity.

The optimized formulation (F6) had shown good in vitro buoyancy and controlled release behavior and hence was finally selected for in vivo study (i.e. gamma scintigraphy), and the results were compared with non-floating microsphere prepared using same polymer (sugar cross linked). Gamma images of the $^{99m}$Tc-labeled F6 and non-floating microspheres are shown in Figure 7. Examination of the sequential gamma scintigraphic images during the study clearly indicated that the F6 remained buoyant and uniformly distributed in the gastric contents throughout the study period of 8 hours. Prolonged gastric retention time (GRT) of more than 6 hours was achieved in all the mice for the formulation F6, which remained buoyant in the stomach for the entire test period. In contrast, nonfloating microspheres showed gastro-retention of less than 2 hours. After swallowing, the floating microspheres adopted a floating position on top of the stomach content. This might be because of the presence of porous structure of microsphere due to emulsifier casein responsible for incorporation of air bubble inside the microspheres. A measurable number of counts of $^{99m}$Tc-tagged F6 for the 8-hour study period showed very good gastro-retentive propensity as the administered microspheres remained floating and distributed in the stomach contents. In case of nonfloating microspheres, the radioactive counts decreased considerably after 2 hours in the stomach.

B(a)P resulted in 100% incidence of forestomach tumors after 10 weeks with an average of 2.19 tumors per mouse compared with corn oil-treated control animals as shown in Figure 8. Mice treated with pure drug and floating microspheres resulted in 30.0% and 55.66% reduction in tumor at first dose. At second dose 42.0% and 86.4% reduction was seen as illustrated in Figure 9. The statistically significant ($P \leq 0.05$) reduction in the number of tumors obtained with formulation F6 as compared with control and plain drug treatments indicates site-specific delivery of doxorubicin through floating dosage forms which results in maintenance of a local concentration of 5-FU for a longer period of time.

In the present study, the potential of porous microsphere as drug carriers for oral delivery was investigated. The method of preparation of microsphere of doxorubicin was found to be simple and reproducible. It was found that porous microspheres showed a desirable high drug content, good flow properties, buoyancy and adequate drug release at pH 4.0; hence, formulation prepared by this method is suitable for controlled and sustained drug delivery. This study shows that porous microsphere could be a useful carrier for doxorubicin.
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