BIO-DISTRIBUTION STUDY OF CROSSLINKED RESEALED ERYTHROCYTES LOADED WITH 5-FLUOROURACIL

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ABSTRACT

5-Fluorouracil (5-FU) is a well-known chemotherapeutic agent used in the treatment of variety of cancers. However, due to exposure of the 5-FU to unwanted sites after biological administration produces serious toxic effects. In the present study, bio-distribution of 5-FU loaded resealed human erythrocytes in albino rats was undertaken. The cellular and in vitro drug release studies were performed. The maximum drug entrapment efficiency was found to be 37.12 % for the selected formulation. In vitro release studies were carried out in phosphate buffer saline (PBS pH 7.2) through dialysis membrane and the drug release estimated spectrophotometrically at 266 nm. A single dose of free 5-FU and 5-FU loaded resealed erythrocytes (30mg/kg) was injected into the tail vein of the rats. After a definite time period (1, 2 and 4 hour) blood samples were obtained from the retro-orbital flexus using a capillary. It was observed that 5-FU loaded resealed erythrocytes could be sustained drug level in blood for a very long time, implying its long systemic retention in the circulation. The 5-FUs were distributed mainly in the liver, kidney and spleen with small quantities in GIT and brain.

KEY WORDS: 5-Fluorouracil, Resealed erythrocytes, bio-distribution.

1. INTRODUCTION

Administration of drugs using cellular carrier mediated drug delivery systems such as resealed erythrocytes (RE) have gained importance due to fact that they are biocompatible, biodegradable and possess long circulation half lives (Jain and Jain,2001; Gopal, 2007). Using RE technique a variety of biologically active compounds (drugs) can be delivered and provides different methods for higher encapsulation efficiency. Highly toxic drugs such as anticancer drugs, the concept of drug masking (avoiding exposure) to unwanted area are important in controlling mortality rates (Murthy, 2001). The concept of drug loaded RE for delivering cytotoxic drugs to a particular site for localized treatment of cancer and thereby decreasing the adverse effect of drug thereby improving its therapeutic index is being considered as a challenge in modern formulation design. 5-Fluorouracil (5FU) is a drug of choice, in treating variety of cancers (David, 2001). The 5FU belongs to an antimetabolite class which mainly acts by noncompetitive inhibition of thymidylate synthase. Adverse effects of this drug are due to restricting the growth of normal proliferating cells and such adverse effects can be avoided by the concept of drug loaded RE (Salonga, 2000).

In the present paper, an optimized formulation of 5-FU was undertaken for the bio-distribution study in albino rats. Efforts have been made to evaluate ex vivo studies along with the tissue distribution at blood, brain, liver, spleen, kidney, lung, heart and gut of 5-FU and 5-FU loaded resealed erythrocytes. Though there are many methods available for the drug loading into erythrocytes, hypotonic osmosis is more promising with high loading efficiency in a lesser efforts. In this research an efforts have been made to load 5FU in human erythrocytes using hypotonic osmosis technique involving preswell dilutional hemolysis. 5FU loaded RE were further crosslinked by glutaraldehyde (GA) and evaluated for physical and cellular characterization.

2. MATERIALS AND METHODS

5-Fluorouracil was received as a gift sample from Cipla Pvt. Ltd., Vikhroli, Mumbai, India. Glutaraldehyde was purchased from S.D. Fine chemicals, Mumbai, India. Phosphate buffer saline, sodium chloride and acetonitrile were purchased from Himedia, India. Packed human blood 500ml of O Rh +ve was obtained from Blood Bank, Cancer Hospital and research center, Navanagar, Hubli, India.

Production of Resealed Erythrocytes: The whole 'O'+ve group blood was centrifuged at 3000 rpm for 10 minutes at 4 ±1°C in a refrigerated centrifuge. The serum and buffy coats were removed by washing three times with phosphate buffer saline (PBS pH 7.4). The washed erythrocytes were diluted with PBS and stored at 4°C until used. To the 10 ml of washed blood cells 40 ml of slightly hypotonic solution (0.6 % W/V NaCl) was added to swell the erythrocytes for 5
min at 0°C. Further, cells were recovered by gentle centrifugation and allowed to swell in presence of 5FU in a 0.4 % w/v solution for 10 min at 0°C. This is followed by restoration of toxicity and incubation at 37°C followed by addition of 2 ml of glutaraldehyde for 10 min. The obtained resealed erythrocytes were washed 3 times with PBS pH 7.4 to remove traces of crosslinking agents (Jain and Jain, 2001).

Drug Entrapment Efficiency: The 5FU inside the RBCs was evaluated by lysing the loaded RBCs with double its volume of distilled water and extracted with acetonitrile and absorbance was measured against similarly prepared blank at 266 nm spectrophotometrically (Jenway, UK). Encapsulation efficiency was determined to find out the total amount of drug which is entrapped in the resealed erythrocytes.

*Ex vivo* experiments: Around 10 ml of blood was collected by carotid bleeding using three animals. The clotting of the blood was prevented by the addition of the 400IU of heparin. Weighed quantity of 5FU was added and stirred using glass rod. The extraction of the drug and analysis of the 5FU was done as per method reported in the literature.

Recovery and % drug was calculated and methods were validated.

Biodistribution study: A single dose of free 5-FU and 5-FU loaded resealed erythrocytes (30mg/kg) was injected into the tail vein of the rats. After a definite time period (1, 2 and 4 hour) blood samples were obtained from the retro-orbital flexus using a capillary. The animals were sacrificed and the heart, kidney, liver, lung, spleen, brain and intestine were collected in buffer solution. Each tissue taken was washed with phosphate-buffered saline (PBS, pH 7.4) and wiped with a filter paper. PBS was then added at a three-fold volume of the weight of the tissue, and the mixture was homogenised. After centrifugation of the homogenate 5-FU was extracted from the tissue with ethyl acetate and isopropyl alcohol (85/15, v/v). The samples were evaporated and dehydrated samples were dissolved in 2ml of mobile phase diluent for subsequent HPLC. Consequently, the amount of 5-FU in each tissue was calculated from the concentration of calculation and tissue weight using standard curve. There is a literature method of calculation of drug concentration according to the following formula (Chen, 2010; Sudha, 2010).

\[
\text{Relative tissue exposure} = \frac{100\% \times (\text{AUC tissue})}{(\text{AUC tissue})} \quad 5 - \text{FU resealed erythrocytes}
\]

\[
\text{Peak ratios} = \frac{100\% \times (\text{Cmax})}{(\text{Cmax})} \quad 5 - \text{FU resealed erythrocytes}
\]

Statistic analysis: The results, obtained by *in-vivo* studies, were statistically analysed using Student’s t-test with a 95% confidence level (p<0.05) and are reported as the mean ± standard deviation (S.D.).

3. RESULTS AND DISCUSSION

In the present study, 5FU was loaded into human erythrocytes using preswell dilutional hemolysis method and crosslinking was done by using two different crosslinking agent viz., glutaraldehyde (GA) and dimethylsulfoxide (DMSO). Details of the formulations and their coding are mentioned in Table 1. Several experiments were conducted to optimize the various parameter such as amount and type of crosslinking agent, various % hypotonic solutions, incubation duration etc., Results of % practical yields are also mentioned in Table 1. The maximum percentage practical yield was found to be around 63.21 % for F1 formulation.

Though during formulation drug to carrier ratio was kept constant for all formulations drug entrapment efficiency was varied with different crosslinking agent. The maximum entrapment efficiency was found for F1 followed by F2. The entrapment efficiencies for F1 and F2 were found to be 37.12±2.0 and 28.73±1.8 % respectively. *In vitro* release of 5FU from the resealed erythrocytes formulations found to depend on type of crosslinking agent. GA crosslinked RE showed slower release rates than the DMSO crosslinked.

Bio-distribution studies were conducted in rats. After injection of 5FU by tail vein in rats blood was collected and extraction of the drug was done and analyzed by HPLC to determine the retention time of 5FU. It was observed that Rf of 5FU was slightly changed from the ex vivo and plain drug HPLC data due to accumulation of metabolites of 5FU in the plasma. As shown in Table 2, after i.v. administration of free 5-FU and 5-FU loaded resealed erythrocytes suspension in rats, the area under plasma concentration-time curve (AUC), the elimination half life (t½), and mean residence time (MRT) were increased several fold for 5FU loaded resealed erythrocytes compared with that of free 5-FU. This may be due to fact that when 5-FU is loaded into erythrocytes, the 5-FU release has been sustained for long time and resealed erythrocytes act as bioreactors thereby, half life increases resulting in increased bioavailability.

The biodistribution of the 5FU loaded resealed erythrocytes formulations and plain 5-FU in various organs in rats were evaluated at distinct durations after i.v. administration. Different organs isolated after injection of 5FU and formulation are as shown in Fig. 1. After 1 hour of injection the amounts of 5-FU in the plasma were slightly different between 5-FU loaded resealed erythrocytes and 5-FU injection in rats were observed. However, at 2nd hour 5-FU
concentration in plasma or tissue samples treated with 5FU loaded resealed erythrocytes and 5-FU injection were found to be significantly different.

It was observed that 5-FU loaded resealed erythrocytes could be sustained drug level in blood for a very long time, implying its long systemic retention in the circulation. The 5-FUs were distributed mainly in the liver, kidney and spleen with small quantities in GIT and brain. Results of bio-distribution of 5-FU scarcely accumulated in the various organs at different time intervals are shown in Table 3. Bio-distribution results indicate that an adequate duration in the circulation of 5-FU resealed erythrocytes should be achievable. It was observed that the concentration of the drug almost in all organs increased with injection % 5FU formulation than injection of the plain drug.

4. CONCLUSION
An anticancer drug 5-fluorouracil was entrapped into human erythrocytes preswell dilutional hemolysis method and crosslinking of the drug loaded erythrocytes were done by glutaraldehyde as well as using dimethylsulphoxide. The maximum % practical yield and highest drug content was observed for the formulations prepared by using GA as cross-linking agent. Bio-distribution results indicate that an adequate duration in the circulation and distribution pattern in various organs altered for 5-FU resealed erythrocytes as compared to plain 5-FU.

5. ACKNOWLEDGMENTS
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Fig. Concentration of 5FU in different organs after injection of (a) plain 5FU and (b) 5FU formulation.

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>Method of loading</th>
<th>Amount of 5FU in mg</th>
<th>Type of crosslinking agent</th>
<th>% Practical yield</th>
<th>% 5FU loading</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>PDH</td>
<td>250</td>
<td>Glutaraldehyde</td>
<td>63.21</td>
<td>37.12±2.0</td>
</tr>
<tr>
<td>F2</td>
<td>PDH</td>
<td>250</td>
<td>DMSO</td>
<td>60.00</td>
<td>28.73±1.8</td>
</tr>
</tbody>
</table>

Table 1: Formulation details of various resealed erythrocytes loaded with 5FU and their results of % practical yield.
Table 2: Amount of 5-FU in Blood after administration of plain 5FU as well as 5FU loaded erythrocytes

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Plain 5-FU</th>
<th>5-FU loaded erythrocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC 0-12 hr</td>
<td>51.21 ± 10.3</td>
<td>144.12 ± 30.12</td>
</tr>
<tr>
<td>T(_{1/2}) (hours)</td>
<td>0.60 ± 0.14</td>
<td>5.14 ± 2.11</td>
</tr>
<tr>
<td>Mean residence time (hours)</td>
<td>0.84 ± 0.05</td>
<td>8.45 ± 1.9</td>
</tr>
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</table>

Table 3: Results of tissue distribution of 5-FU after administration of plain 5FU as well as 5FU loaded erythrocytes

<table>
<thead>
<tr>
<th>Time in hours</th>
<th>Brain µg/g of tissue</th>
<th>Liver µg/g of tissue</th>
<th>Spleen µg/g of tissue</th>
<th>kidney µg/g of tissue</th>
<th>GIT µg/g of tissue</th>
<th>Brain µg/g of tissue</th>
<th>Liver µg/g of tissue</th>
<th>Spleen µg/g of tissue</th>
<th>kidney µg/g of tissue</th>
<th>GIT µg/g of tissue</th>
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<tr>
<td>1</td>
<td>4.1±0.2</td>
<td>8.2±0.7</td>
<td>9.7±0.9</td>
<td>121±92</td>
<td>9.1±0.19</td>
<td>3.7±0.01</td>
<td>5.2±0.2</td>
<td>7.8±0.02</td>
<td>80.45±2.18</td>
<td>3.4±0.01</td>
</tr>
<tr>
<td>2</td>
<td>6.2±0.7</td>
<td>11.5±1.9</td>
<td>11.3±2.01</td>
<td>145±27</td>
<td>10.4±2.1</td>
<td>4.8±2</td>
<td>20.7±2</td>
<td>12.4±0.11</td>
<td>94.7±3.71</td>
<td>8.7±0.07</td>
</tr>
<tr>
<td>4</td>
<td>5.8±0.1</td>
<td>7.2±1.2</td>
<td>6.6±0.09</td>
<td>80.6±54</td>
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<td>4.8±0.01</td>
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<td>97.5±11.21</td>
<td>9.7±2.1</td>
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REFERENCES


