IJBPAS, December, 2021, 10(12): 4431-4444

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

DESIGN AND EVALUATION OF CAPECITABINE RESEALED ERYTHROCYTES BY PRESWELL AND DILUTION TECHNIQUES

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Received 20th Feb. 2021; Revised 20th Mar. 2021; Accepted 16th April. 2021; Available online 1st Dec. 2021

https://doi.org/10.31032/IJBPAS/2021/10.12.5761

ABSTRACT

Resealed erythrocyte is novel drug delivery system mainly used for targeting specific tissues with drug. This drug delivery system is having number of advantages over the other novel drug delivery system in case of the diseases like cancer. There are various methods used for preparation of resealed erythrocyte. In this study, we have prepared Resealed erythrocytes by two techniques, Dilution technique and preswell dilution technique by adding cross linking agent, glutaraldehyde due to which % encapsulation efficiency was increased and the results were obtained. With these results, we found that, in vitro drug release followed first order kinetics with sustained release. The % encapsulation efficiency was more with the formulations prepared by preswell technique as compared to dilution technique. The hematological parameters were satisfactory. The other parameters like particle size, zeta potential, osmotic shock, osmotic fragility, turbulent shock studies, stability study were tested for the optimized formulations. This drug delivery system has exclusive advantages over conventional drug delivery systems and hence proved to be potential in clinical point of view.

Keywords: Dilution, % Encapsulation Efficiency, Glutaraldehyde, Preswell, Resealed Erythrocytes

INTRODUCTION

Erythrocytes are used as highly biodegradable and biocompatible drug carriers with the advantage of selective transport of drug delivery to RES organs **[1]**. These erythrocytes based drug delivery systems are efficient in maintaining the active drugs in circulation, controlled and prolonged release and selective targeting of drugs, enzymes, peptides or hormones to specific organs, tissues cells **[2]**. In 1979, the term "carrier erythrocytes" was used first time to describe drug loaded erythrocytes. These carrier erythrocytes are given intravenously for the site specific delivery of antineoplastic agents, cardiovascular agents, vitamins, steroids, antibiotics, etc **[3]**. Besides this, they have other advantages of having no undesired immune response against encapsulated drugs, protection of loaded drugs from inactivation by endogenous enzymes, modification of pharmacokinetic and pharmacodynamic parameters of the drug, possibility of reducing the side effects of drugs with some drawbacks like leakage of entrapped drug, storage problem of drug loaded erythrocytes, possibility of contamination due to origin of blood, instrument, etc. **[3]**. Capecitabine, a fluoropyrimidine carbamate is a prodrug which enzymatically converted to 5 flurouracil in the body tissues after oral or parental administration. The drug was approved for colorectal and metastatic breast cancer. It inhibits DNA synthesis and slows down the growth of tumour **[4]**.

It is readily absorbed from G. I. tract and has short elimination $t_{1/2}$ (38-45 min.). It is available in 150mg and 500mg dose with recommended dose of 1250 mg/m^2 bid for two weeks and repeated on 22nd day. Capecitabine is associated with the adverse effects like bone marrow depression, cardiotoxicity, diarrhea, hand- foot syndrome, nausea, vomiting and dermatitis etc. hence the attempts were made to formulate the Capecitabine in controlled / sustained release dosage form and especially in multiparticulate drug delivery system such as microspheres, nanoparticles, beads, etc. thereby reducing the side effects and increasing bioavailability **[5, 6]**. The main objective of this study is to remain the drug at its therapeutic limits for longer time. Most of the anticancer drugs were loaded into erythrocytes because of the biocompatibility and biodegradability, longer $t_{1/2}$ and reduction in adverse effects **[4]**.

It has been observed that, carrier erythrocytes treated with certain substances alters the properties of drug loaded erythrocytes **[3]**. The drug loaded erythrocytes treated with cross linking

agent like glutaraldehyde makes the formulation more thermostable and releasing the drug slowly with increased osmotic resistance and turbulence resistance.

MATERIALS

All the materials used were of analytical grade. Capecitabine was received as gift sample from Hetero Labs, Hyderabad. Other chemicals such as PBS pH 7.4, acetonitrile, normal saline, glutaraldehyde (purchased from Lobe chemie) were of analytical grade.

METHODS

Preparation of human erythrocytes-

The whole 'O' blood samples were obtained from registered blood bank (Krishna Hospital, Karad, Maharashtra). It was centrifuged at 3000 rpm for 5 min. at 4± 1°C in cooling centrifuge (Plastocrafts). The serum and buffy coats were removed by 3 times washing with phosphate buffer saline pH 7.4. The washed erythrocytes were diluted with PBS and stored at 4° C until use.

1. **Preparation of Capecitabine loaded erythrocytes by dilution technique:-**

For the preparation of resealed erythrocytes human blood (O Blood group) stored under refrigerated conditions was used. A 50% v/v suspension of the washed erythrocytes was prepared in cold saline and an aliquot of this suspension was added to a flask containing the cold haemolysing medium. The haemolysing medium contained 25 ml of 0.3% w/v NaCl solution and 10 ml of 1% w/v drug solution and then incubated at 25°C in an isotonic solution (0.9% w/v NaCl) to reseal them again. The suspension was washed 3 times with phosphate buffer saline and then suitably diluted with PBS and stored at 4°C in refrigerator. By using the above method, 8 more batches of resealed erythrocytes were prepared by using different concentrations of drug and cross linking agent, glutaraldehyde (F1 to F9). The whole experiment was carried out by maintaining the temperature at 0-4°C **[7, 8]**.

2. **Preparation of Capecitabine loaded erythrocytes by Preswell dilution technique:**

For the preparation of resealed erythrocytes human blood (o blood group) stored under refrigerated conditions was used. A hypotonic solution (0.3% w/v NaCl solution) was prepared and an aliquot of this solution was added to a flask containing 50% v/v suspension of RBC's (erythrocytes) up to the point of haemolysis. To the swelled RBC's, 10 ml of 1% w/v drug solution was added and isotonicity of swelled erythrocytes was retained by adding hypertonic solution (1.3% w/v NaCl solution) to reseal the

membrane by incubating at 0°C for 5 min and then gently centrifuged to remove unentrapped drug solution on the surface of the membrane.

The suspension was washed 3 times with phosphate buffer saline pH 7.4 and then suitably diluted with PBS and stored at 4°C in refrigerator (F1). By using the above method 8 more batches of resealed erythrocytes were prepared by using different conc. of drug and glutaraldehyde solutions (F1 to F9). The whole experiment was carried out by maintaining the temperature at 0-4°C **[7, 9]**.

Optimization of batches:-

For both the techniques (Dilution and preswell), nine+ nine batches were prepared using different concentrations of drug (Capecitabine) and cross linking agent (glutaraldehyde). The batches were optimized on the basis of their % encapsulation efficiency. Out of 9, 1 batch of each technique was optimized and processed further for lyophilization.

Procedure for Lyophilization:-

Lyophilization was carried out immediately after the preparation of formulation. The suspension of Capecitabine encapsulated erythrocytes was transferred into glass vials (100 ml capacity, Yantai Uech Pharmaceutical Package Co., Ltd, China). The rubber closures (Apipharma, India) were positioned as half-closed. The solutions were frozen at – 40 °C for 6 h in deep freezer (Polar 530V, Angelantoni Industries India). The prefrozen vials were lyophilized in Labconco FreeZone 2.5 lyophilizer at shelf temperature - 40 °C under vacuum 0.016 hPa for 48 h (condenser temperature – 50 °C). Secondary drying was performed at 5 °C for 2 h. After freeze drying, the dry mass was broken into free flowing powder using glass rod and then packaged in amber color glass vials and stored in refrigerator until use **[10]**.

Physical Characterization:-

1. ***Drug content:-***

Drug content of the cell determines the entrapment efficiency of the method used. The process involves deproteinization of packed and loaded cells (0.5ml) with 2 ml acetonitrile and centrifugation at 2500 rpm for 10 min. the clear supernatant was analyzed for the drug content spectrophotometrically **[11]**.

2. Zeta potential and particle size analysis:-

The size distribution of the sample was determined by using the particle size and zeta potential analyzer (Horiba SZ-100) equipped with a dry accessory system in which a drop of sample was diluted with ten times double distilled water. The sample was taken in cuvettes and then analyzed **[12]**.

3. Haematological indices:-

The mean corpuscular volume (MCV), mean corpuscular haemaglobin (MCH) and mean corpuscular haemaglobin content(MCHC) of unloaded erythrocytes, capecitabine loaded erythrocytes by dilution technique and capecitabine loaded erythrocytes by preswell dilution technique were determined using Nihonkohden (Model-MEK9100k) haematology autoanalyser **[13, 14]**.

4. In vitro drug release:-

Release of capecitabine from resealed erythrocytes was studied using the dialysis method at 37 ± 2°C and was compared with the pure drug (capecitabine) solution. The dialysis bags (HIMEDIA) were first hydrated for 30-60 min with PBS (pH 7.4) and resealed erythrocytes (500 µL) were loaded carefully using a syringe without puncturing the dialysis membrane. Then, the tubes were immersed in 100 ml of release medium PBS (pH 7.4). While stirred the release medium using the magnetic stirrer at 150 rpm/min, 2 ml samples were withdrawn at predetermined time intervals and diluted upto 5ml with PBS. From the release medium and the same volume was replaced with fresh medium to maintain sink conditions. The sample was analyzed at 240 nm using the UV-visible spectrophotometer (Shimadzu 1900, Japan) **[4, 15, 16]**.

Cellular Characterization:-

1) Osmotic shock:-

Capecitabine loaded erythrocytes were incubated with distilled water for 15 min followed by centrifugation at 3000 rpm for 10 min and may cause the release of drug, and it was estimated using the UV-Visible spectrophotometer at 240 nm **[4, 17]**.

2) Osmotic Fragility:-

It is the reliable parameter for the *in-vitro* evaluation of carrier erythrocyte with respect to shelf life, in vivo survival and the effect of encapsulated substances. 10 mg of drug loaded resealed erythrocytes suspended in isotonic saline (10 ml) and was incubated separately in stepwise decreasing concentration of sodium chloride solution (0.9%w/v to 1%w/v) at 37±2°C for 10 minutes, followed by centrifugation at 2500 rpm for 10 min and supernatant was examined for drug content. It is based on resistance of cells to haemolysis in decreasing concentration of hypotonic saline **[9, 14, 16]**.

3) Turbulent shock studies:-

The erythrocyte suspension was passed through 23 standard of measurement needle several times at a flow rate of 10 ml/min. and % haemolysis was calculated. The number of passes were plotted against %

haemolysis for both loaded and unloaded erythrocytes **[8, 16]**.

4) Shape and surface morphology:-

A JEOL JSM-6380 LA scanning electron microscope (Jeol Ltd., Tokyo, Japan) was used to evaluate morphological differences normal and Capecitabine loaded erythrocytes. The cell were fixed in buffered gluteraldehyde, and rinsed 3 times for 5 min in phosphate buffer and post-fixed in osmium tetroxide for 1 h. Then, samples were rinsed with distilled water and dehydrated using a graded ethanol series: 25, 50, 75, and 100% 10 min for each. Finally, samples were rinsed in water, removed, mounted and then viewed using SEM (Harisa *et al*., 2012) **[13]**.

Stability studies:-

There is no specific method for stability of Resealed erythrocytes. Here, the two optimized batches of both the techniques (Dilution and preswell techniques) were tested after lyophilization for stability studies at 4°C, 45% RH, at room temperature and at 37˚C ± 2˚C, 65% ± 5% RH. The thermostatic humidity control oven was used to maintain humidity. After 15 days and one month, both the formulations were subjected for the drug content study **[10, 18]**.

RESULT AND DISCUSSION

1. Drug content:-

The drug content was estimated in all 9+9 batches of Capecitabine with cross linking agent. The encapsulation efficiency of drug was increased with increased conc. of cross linking agent but up to certain limit. The maximum entrapment efficiency was 86.40 % (F7 of preswell technique) and lowest was 50% (F3 of preswell technique) and 81.15 % (F7 of dilution technique) and lowest was 21.47 % (F3 of dilution technique) as shown in the graph. In vitro drug release studies were based on the drug content present in each formulation **(Figure 1)**.

From **Figure 1** results it can be concluded that F7 formulations of both the methods (Capecitabine 5% and Glutaraldehyde 0.5%) showed higher % encapsulation efficiency.

2. Zeta potential:-

The Zeta potential values of the optimized formulations were found to be -14.4 mV for F7 of dilution technique and -18.0 mV for F7 of preswell technique. These values were in the range of zeta potential values of the normal erythrocytes. By this it is concluded that we can make stable formulations of Capecitabine resealed erythrocytes.

Particle size:-

The average particle size of the optimized formulations was found to be 2.286 µm for F7 of dilution technique and 3.639 µm for

F7 of preswell technique. These results showed that there were no much differences between particle size of loaded and unloaded erythrocytes. The particle sizes were within the prescribed limits to prepare resealed erythrocytes.

3. Haematological indices:-

Here, the hematological parameters, such as MCV, MCH and MCHC were tested. These parameters determine the effect of drug encapsulation process on the hematological properties of the erythrocytes. **Table 1** represents the mean hematological parameters of the Capecitabine loaded erythrocytes obtained with different Capecitabine and glutaraldehyde concentrations. There were no significant differences in haematological parameters of loaded erythrocytes as compared with control but little difference was seen with optimized formulation by Preswell technique and optimized formulation by dilution technique. This result revealed that there was more haemoglobin loss during loading procedure which was even more in dilution technique than in Preswell technique. It indicated that Preswell technique was less destructive procedure than dilution technique.

4. In- vitro drug release:-

The in vitro drug release from capecitabine solution, optimized capecitabine-loaded erythrocytes were studied at 37°C ± 2 in PBS buffer. When the release of plain Capecitabine was evaluated using dialysis bag (Himedia) as barriers, ~100% drug was available in the receiver chamber after 6 hrs. The overall percent drug release in 36 hrs ranged from 13 – 96 %. The percent cumulative drug release for F7 formulation of dilution technique (D sample) was 69.65 % after 24 hrs and 72.4 % after 36 hrs. The same for F7 formulation of preswell technique (P sample) was 87.34 % after 24 hrs and 96.34 % after 36 hrs. Both are the sustained release formulations and the release of capecitabine from carrier cells follows first order kinetics. This indicated that the formulation prepared by preswell technique showed more % entrapment efficiency as compared to that by dilution technique **(Figure 2)**.

Stability studies:-

The stability studies of Capecitabine loaded erythrocytes were carried out at 4^0C, 45% RH, room temperature and at 37^0C ± 2^0C, 65% ± 5% RH for a month. The results of drug content study showed that 4^0C and 45 % RH is the ideal storage condition for both the formulations.

Cellular Characterization:-

1.Osmotic shock:-

Osmotic shock was carried out for the formulation F7 of both techniques to evaluate the ability of resealed erythrocytes to withstand stress and maintain their

integrity as well as appearance. When drug loaded erythrocytes were incubated with distilled water the cells were completely ruptured and there was complete release of hemoglobin from the cell. This indicates that there was complete lysis of the erythrocytes when both formulations were incubated with water for osmotic shock study.

2. Osmotic fragility:-

Osmotic fragility test is the indicator of possible changes in the cell membrane integrity caused due to loading procedure. It measures the resistance of these cells to changes in the osmotic pressure of surrounding media. In hypotonic medium the cell membrane breaks which allows hemoglobin to escape from the cell. Generally, hemolysis starts at 0.4-0.5% and completes at 0.3% NaCl concentration.

Here, in Preswell technique, the loaded erythrocytes have greater fragility than normal erythrocytes. As per the findings of other researchers (Talwar and Jain), the shape of graph of loaded erythrocytes was sigmoidal and that of normal cells was nearly linear. The similar results were found in our study as shown in **Figure 3**. The lower resistance of carrier cells to osmotic changes indicates the loss of integrity of the red cell membrane naturally (M. Hamidi *et al*). In osmotic fragility test for dilution technique, the similar results were not observed which means shape of graph of % hemolysis of loaded erythrocytes was not exactly sigmoidal as compared with the shape of graph of loaded erythrocytes in Preswell technique as shown in **Figure 4**.

From this, we could say that osmotic fragility of loaded erythrocytes in Preswell technique can resist the changes of osmotic pressure in surrounding media more as compared to the loaded cells in dilution technique.

3. Turbulent shock studies:-

This test is the measure of simulating distribution of drug loaded cells during injection. Also this test is the indicator of mechanical strength of cell membrane integrity in the circulation. When drug loaded cells passed through 23 mm guage needle, the hemoglobin content were slightly more than that in normal cells indicating slight increase in the fragility of loaded erythrocytes.

In our findings in Preswell technique, though turbulence shock was greater for drug loaded erythrocytes, there was no significant difference in % hemolysis of loaded and normal erythrocytes as seen in **Figure 5**. In dilution technique also, no significant difference was observed in % hemolysis of loaded and normal erythrocytes shown in **Figure 6**.

4. Shape and surface morphology:-

Figure 7, 8.

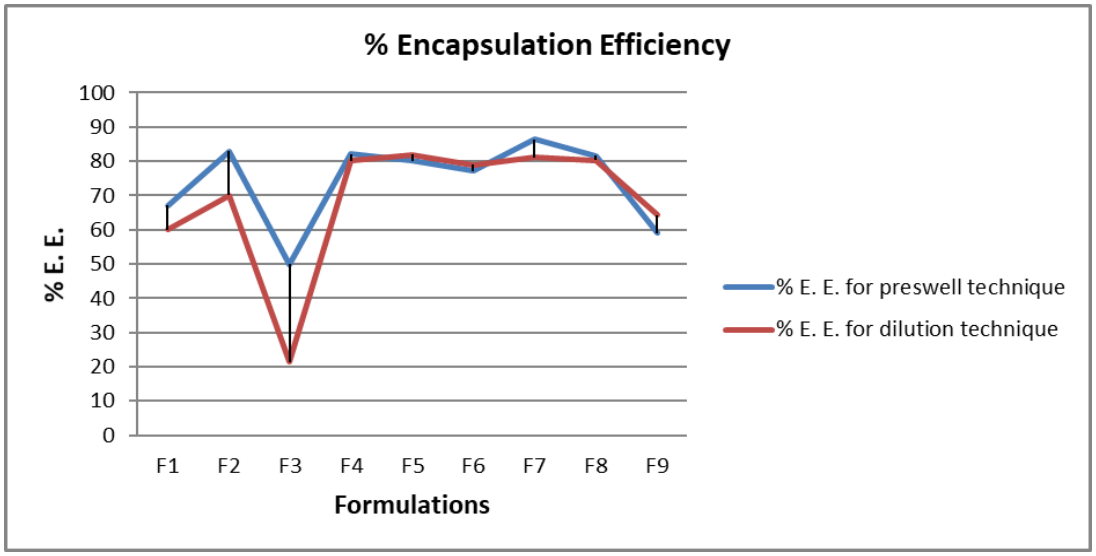

Figure 1: Comparison of Drug Entrapment Efficiency between Different Formulations of Capecitabine Resealed Erythrocytes prepared by Preswell technique and dilution technique
Values are mean±SD of triplicates

Table 1: Haematological Parametres of optimized formulations compared with control sample (sham encapsulated erythrocytes)

Sample	*MCV (fL)*	*MCH (Pg)*	*MCHC (g/ dL)*
Control	**79.6**	**29.0**	**32.0**
F7 of dilution technique	**76.6**	**20.4**	**22.6**
F7 of preswell technique	**78.4**	**21.8**	**25.7**

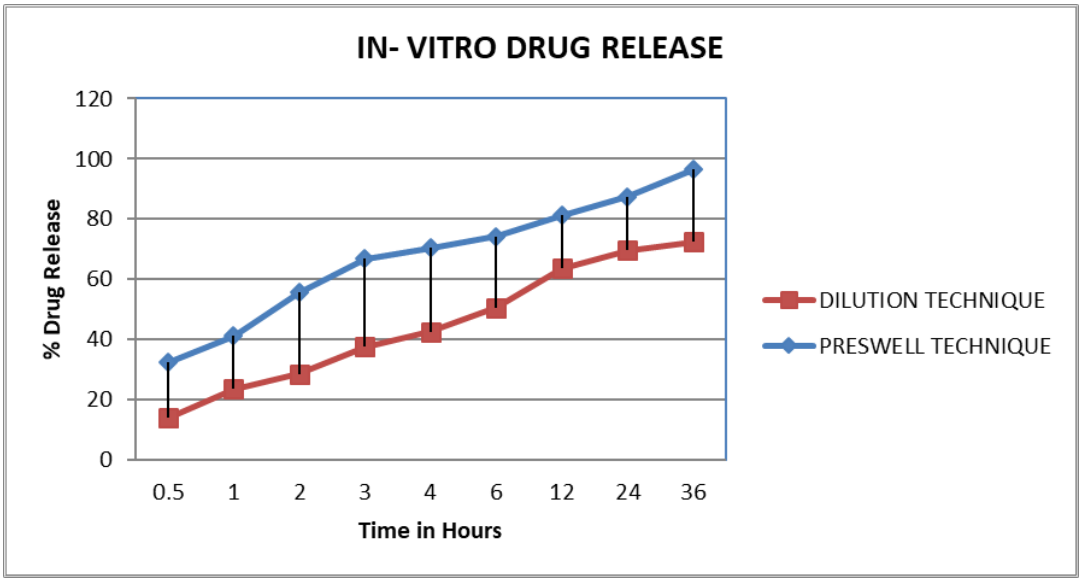

Figure 2: Invitro Drug Release Studies for optimized Formulations of Capecitabine Resealed Erythrocytes
Values are mean±SD of triplicates

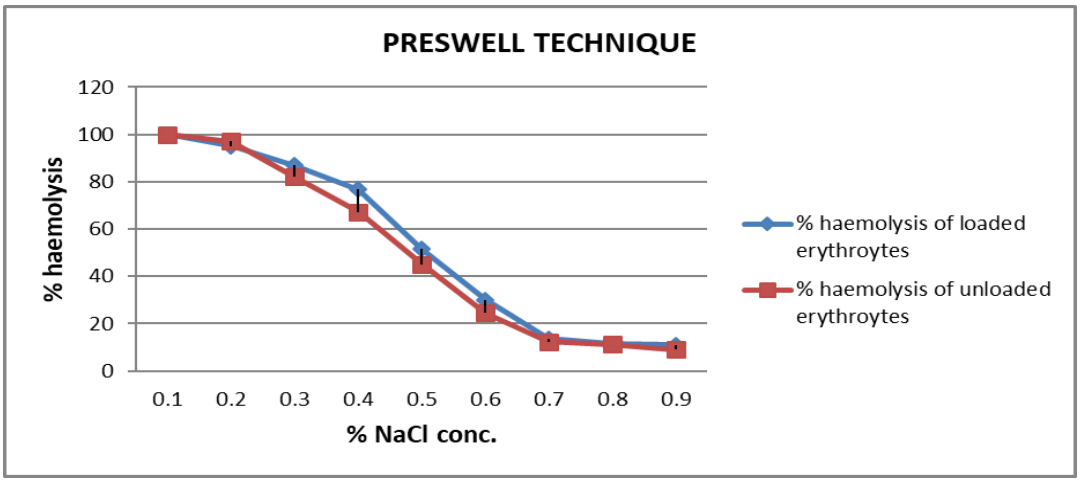

Figure 3: Osmotic fragility of loaded and unloaded erythrocytes by Preswell technique
Values are mean±SD of triplicates

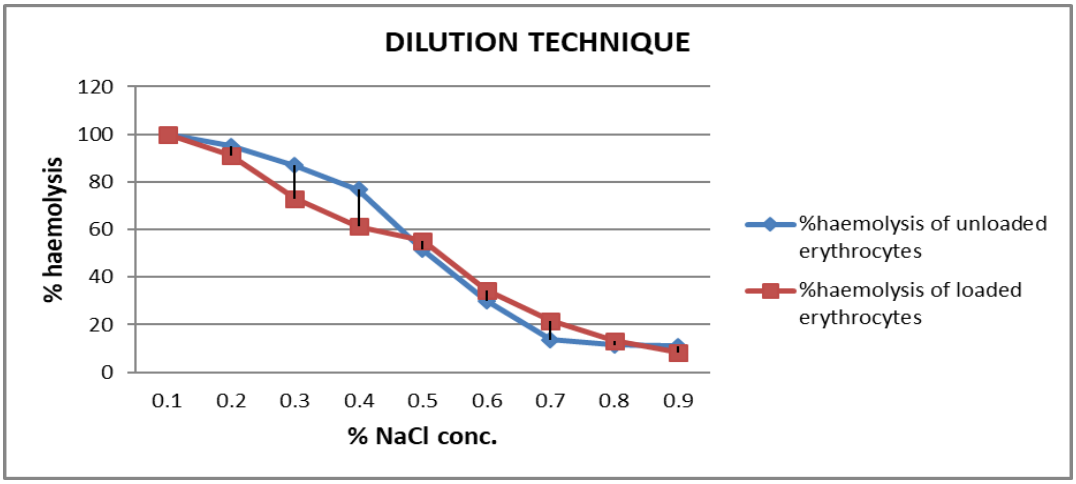

Figure 4: Osmotic fragility of loaded and unloaded erythrocytes by Dilution technique
Values are mean±SD of triplicates

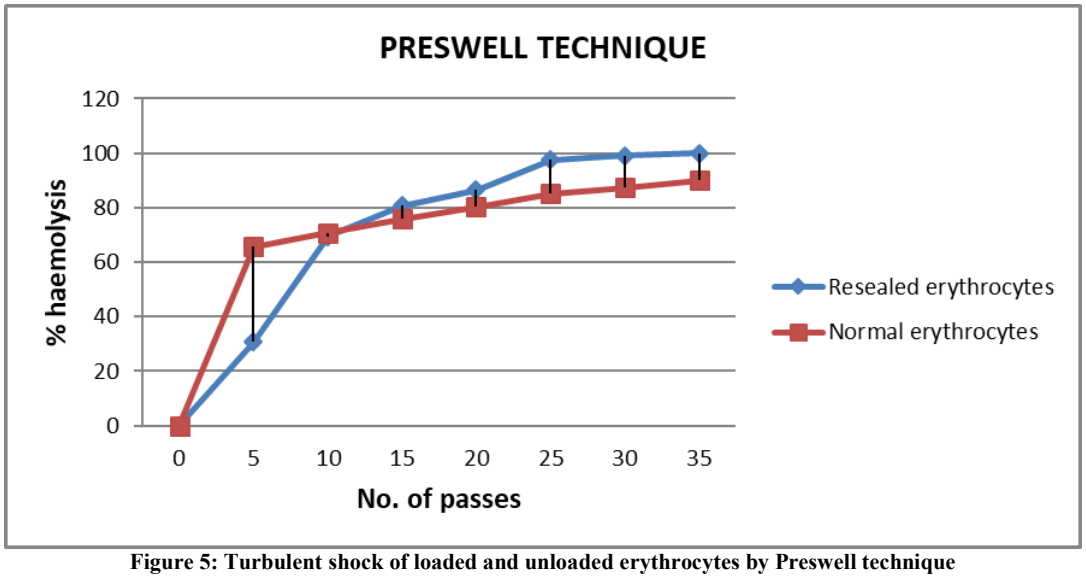

Figure 5: Turbulent shock of loaded and unloaded erythrocytes by Preswell technique
Values are mean±SD of triplicates

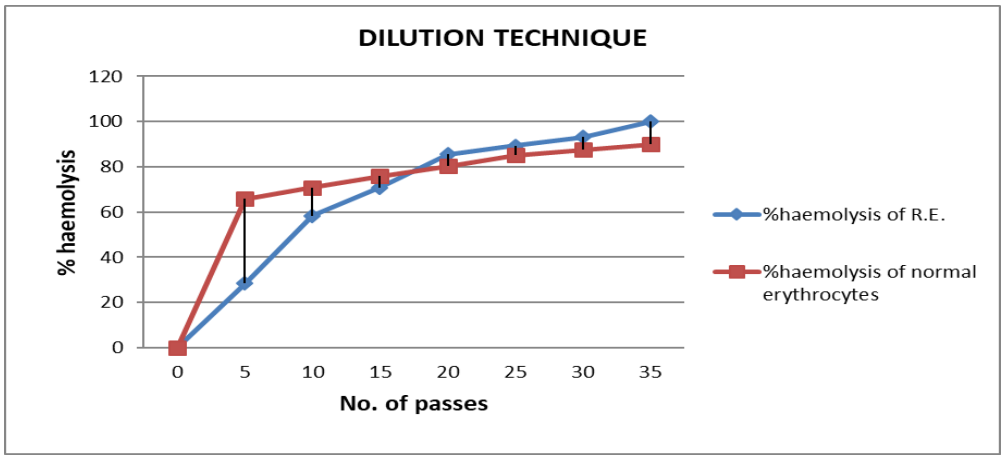

Figure 6: Turbulent shock of loaded and unloaded erythrocytes by Dilution technique
Values are mean±SD of triplicates

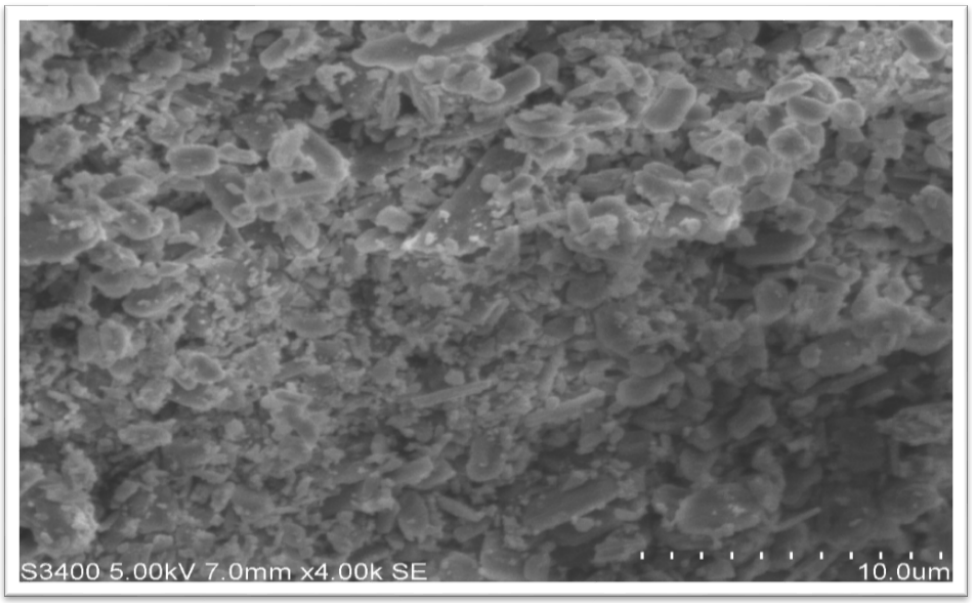

Figure 7: SEM image of drug loaded erythrocytes prepared by Preswell technique

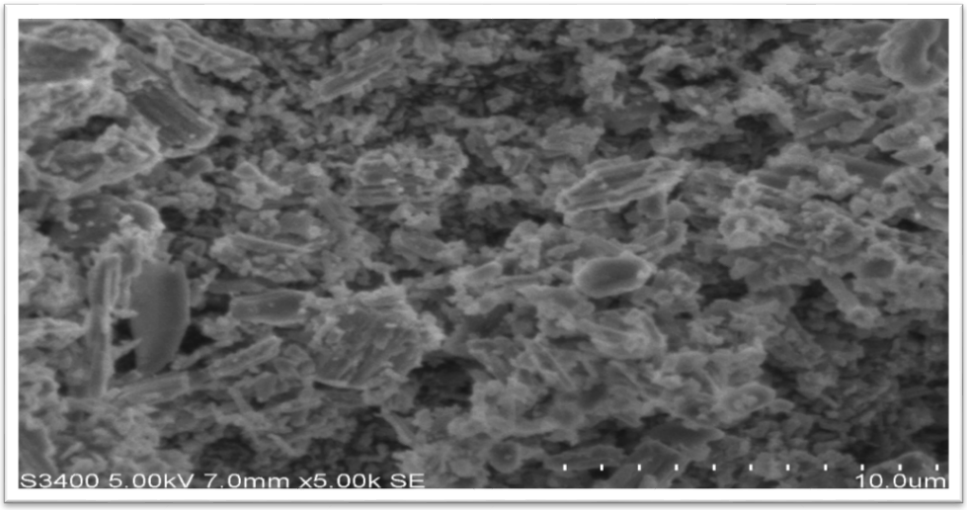

Figure 8: SEM image of drug loaded erythrocytes prepared by Dilution technique

According to study by **Garin *et al,* 1996,** the erythrocytes maintain their normal biconcave discoid shape after loading procedure **[7]**. According to the literature, loaded cells may or may not have changes in shape or size. In our study, no significant change was seen after loading process. This was observed especially in loading process by Preswell technique but there were so many irregularities in shapes and sizes of loaded cells prepared by dilution technique. This indicated the destructions during loading process. Both the images suggested that Preswell technique was less destructive process as compared with dilution technique.

CONCLUSION

Capecitabine, an anticancer prodrug of 5 Fluorouracil, is the suitable carrier for resealed erythrocytes. The formulations were prepared by dilution technique and preswell technique and the results were studied. The % encapsulation efficiency of drug was more with preswell technique than with dilution technique. Addition of Glutaraldehyde as a cross linking agent to the formulation at suitable concentration increased the encapsulation efficiency of Capecitabine. The haematological parameters were satisfactory. Capecitabine released from loaded erythrocytes obeyed first order kinetics and sustained release. The formulation by preswell method released 96.34% drug after 36 hrs and that by dilution method was 72.4%. Both formulations of resealed erythrocytes were evaluated for drug content, osmotic shock, osmotic fragility and turbulence shock studies. In osmotic fragility studies, osmotic fragility of loaded erythrocytes in Preswell technique can resist the changes of osmotic pressure in surrounding media more as compared to the loaded cells in dilution technique and in turbulent shock studies, there was no significant difference in % hemolysis of loaded and normal erythrocytes for both techniques. SEM studies showed that Preswell technique is less destructive than dilution technique. The present work showed that the targeting efficiency of drug loaded erythrocytes over free drug is more which may reduce the dose of a drug required for the therapy and also reduce the systemic side effects. This also showed that, designing of Capecitabine loaded erythrocytes is better for site specificity and prolonged release therapy. In future, in- vivo studies need to be carried out to check the safety, efficacy and bioavailability of the formulation.

ACKNOWLEDGEMENT

The authors would like to thank Krishna Institute of Medical Sciences and Research Centre, Karad and Krishna Institute of Pharmacy, Karad for providing the necessary liabrary facilities, laboratory

facilities, blood bank, instrumental facilities. We wish to thank Hetero Drugs, Hyderabad, Telangana for providing Capecitabine as free gift sample.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

FUNDING SOURCE

The authors are thankful to Krishna Institute of Medical Sciences Deemed to Be University Karad for providing necessary financial assistance.

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