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Formulation and *in-vitro* evaluation of Prednisolone engineered erythrocytes

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ABSTRACT

In the present investigation erythrocytes obtained from healthy volunteers were loaded by prednisolone using preswell dilution and dilution technique with two different cross-linking agents, glutaraldehyde and dimethylsulphoxide. Carrier erythrocytes, having acceptable loading parameters showed increased percentage drug content with the addition of cross-linking agents. In vitro release studies showed drug release followed zero-order kinetics, hemoglobin content was found to be satisfactory and osmotic fragility study indicated increased drug entrapment efficiency was found at 0.3%w/v concentration of sodium chloride (hypotonic solution). This drug delivery is endowed with several exclusive advantages and hence holds potential for further research and clinical application.

Keywords: Resealed erythrocytes, Preswell dilution technique, Dilution technique; Prednisolone, glutaraldehyde and dimethylsulphoxide.

INTRODUCTION

The current pharmaceutical scenario is aimed at development of drug delivery systems with maximum therapeutic benefits for safe and effective management of disease(s). The emerging advances in the development of novel drug delivery technologies are likely to have significant impact on drug industry. There are two desirable properties for a drug carrier, to selectively direct a drug to a target tissue and to protect drug from premature bioinactivation. Amongst the various carriers used for targeting of drugs to various body tissues, the cellular carriers meet

several criteria desirable in clinical applications, among the most important being biocompatibility of carrier and its degradation products. Leukocytes, platelets and erythrocytes have been proposed as cellular carrier systems. Among these, the erythrocytes have been the most investigated and have found to possess great potential in novel drug delivery. Resealed erythrocytes are gaining more popularity because of their ability to circulate throughout the body, biocompatibility, zero-order release kinetics, reproducibility and ease of preparation. Most of the resealed erythrocytes used as drug carriers are rapidly taken up from blood by macrophages of reticuloendothelial system (RES), which is present in liver, lung, and spleen of the body. Among the methods proposed by investigators for drug loading intact erythrocytes such as antineoplastic agents, angiotensin-converting enzyme inhibitors, systemic corticosteroids and prodrugs.

Prednisolone is a corticosteroid of the class glucocorticoids and is a drug of choice for systemic inflammatory and immunosuppressive effects. It is a useful drug in the treatment of respiratory, liver diseases, organ transplantation, cancer and autoimmune diseases. In most of the cases, larger doses and prolonged therapy is required, which leads to high incidence of adverse side effects, some of which are potentially life threatening.

The drug is also extensively bound to plasma proteins and only fraction of corticosteroid that is unbound can enter cells to mediate corticosteroid effect. These inherent drawbacks of prednisolone demanding an alternative drug delivery system to conventional drug delivery systems. Hence an attempt has been made to provide an alternative drug delivery system of prednisolone in the form of resealed erythrocytes to reduce adverse effects and to enhance therapeutic efficacy of drug.

MATERIALS AND METHODS

Materials

Prednisolone was obtained as gift sample from Wyeth Lederle Ltd., Goa. Other chemicals like sodium chloride, potassium dihydrogen orthophosphate etc and solvents like Acetonitrile, DMSO etc were laboratory or HPLC grades as required.

Methods

Isolation of Human Erythrocytes

The whole O group blood obtained from registered Blood Bank (red cross blood bank centre, Guntur, Andhrapradesh, India) was centrifuged at 3000 rpm for 5 minutes at 4±1°C in a refrigerated centrifuge (Plastocrafts, Bangalore). The serum and buffy coats were removed by washing 3 times with phosphate buffer saline (PBS: NaCl, 150mmol/l; K2HPO4, 5mmol/l; pH 7.4). The washed erythrocytes were diluted with PBS and stored at 4°C until used.

1. Encapsulation of prednisolone in erythrocytes by preswell dilution technique

For the preparation of resealed erythrocytes human blood (o blood group) stored under refrigerated conditions was used (**Table no.1**). 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 2 more batches of resealed erythrocytes were prepared by using 2 different cross linking agents glutaraldehyde solution (2 ml of 10% v/v glutaraldehyde solution) (F2) and DMSO (dimethylsulphoxide) solution (2ml of 10% v/v DMSO solution) (F3) to compare the effect of cross linking agent. The whole experiment was carried out by maintaining the temperature at 0-4°C.

Table No.1: Preswell dilution technique

Batch	Formula
F1	50% v/v of Erythrocytes + 100 mg Drug
F2	50% v/v Erythrocytes + 100 mg Drug + 10% v/v Glutaraldehyde solution
F3	50% v/v of Erythrocytes + 100 mg of Drug + 10% v/v of DMSO solution

2. Encapsulation of prednisolone in erythrocytes by dilution technique

For the preparation of resealed erythrocytes, human blood (O Blood group) stored under refrigerated conditions was used (**Table. No.2**). A 50% v/v suspension of the washed erythrocytes was prepared in cold saline and an aliquot of this suspension was added slowly 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 (F4). By using the above method, 2 more batches of resealed erythrocytes were prepared by using 2 different cross linking agents, glutaraldehyde (F5) and dimethyl sulphoxide (F6). The concentration used was same as mentioned in preswell dilution technique. The whole experiment was carried out by maintaining the temperature at 0-4°C.

Table No.2: Dilution technique

Batch	Formula
F4	50% v/v of erythrocytes + 100 mg
F5	50% v/v of erythrocytes + 100 mg of Drug + 10% v/v Glutaraldehyde solution
F6	50% v/v of Erythrocytes + 100 mg of Drug + 10% v/v of DMSO solution

The F1, F2, F3, F4, F5 & F6 obtained were lyophilized by using lyophilizer to convert them into dry form in frozen state at extremely low pressure and has great potential to extend the shelf-life of drug and drug carriers. Dry products with sufficient long-term stability and reconstitution properties were obtained by lyophilizing the formulation.

Procedure for lyophilization:

Lyophilization was carried out immediately after the preparation of the formulation. The suspension of drug encapsulated resealed erythrocytes was taken in a 250ml beaker and frozen to a temperature of -40^0C in the bath compressor of the lyophilizer (Lyodel). The frozen product obtained after 2hrs in the bath was then transferred to the vaccum trap of the lyophilizer for drying under vaccum and subjected to bath temperature of -40^0 C under a pressure of 10-2 torr for 4hrs. After freeze-drying, the lyophilized product was obtained in the form of dry mass. This mass was broken gently using a glass rod to obtain a free flowing powder of lyophilized product. Lyophilized product was packed in amber coloured vial and preserved in freeze until use.

I. Physical characteriztion

Drug Assay

Glucocorticoid analogues were determined by HPLC on boiled samples according to the method described by Magnani et. al. Briefly, 10 mg of drug loaded resealed erythrocytes (suspensions) from each batch was diluted up to 100 ml with doubly distilled water, boiled for 5 min and filtered through 0.22 µm filters. HPLC determinations were performed with 5 µm Supelcosil (C-18 column/ 25 cm x 4.6 mm internal diameter) protected by guard column. The Mobile phase consisted of two eluents: Buffer A containing 10 mM KH2PO4, pH 5.0 and Buffer B containing Buffer A + 70% (v/v) acetonitrile adjusted to pH 5.0. The flow rate = 1.0 ml / min and detection was at 239 nm. 20µl of the sample was injected in the chromatograph.

Shape and surface morphology

Particle size and shape analysis was done by photomicrograph using microtek optical microscope. Small amount of resealed erythrocyte suspension was placed on a clean slide; pictures of resealed erythrocytes were taken by random scanning of the slide. Finally, diameter of about 10-20 resealed erythrocytes was manually measured from photomicrographs of each batch.

Drug Release studies

To exploit the release kinetics of prednisolone from carrier erythrocytes (lyophilized product) containing 10 mg of prednisolone were taken and suspended in 20 ml of isotonic saline, for diffusion in a inverted measuring cylinder using 100 ml of pH 7.4 phosphate buffer as the diffusion medium in a beaker. The mouth of the measuring cylinder was covered with cellophane paper, which acts as semi permeable membrane and fitted carefully such that paper was fixed to the mouth of measuring cylinder. The measuring cylinder was held in position by means of clamps. The time at which diffusion was initiated was noted and 5 ml of diffusate was withdrawn with pipette at various time intervals of 30 min, 1, 2, 3, 4, 6, 7, 8, 12, and 16 hrs, and replaced by the same volume of phosphate buffer saline. These samples were filtered through 0.45 µm membrane filter. The samples were deproteinized with Acetonitrile diluted suitably. The drug was estimated in each batch by UV spectrophotometer (Shimadzu Corporation, Japan) at 239 nm.

II. Cell related characterization

Osmotic Fragility

To evaluate the resistance of erythrocytes membrane against the osmotic pressure changes of their surrounding media, drug loaded resealed erythrocytes suspended in isotonic saline 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.

Osmotic Shock:

Osmotic shock describes a sudden exposure of drug-loaded erythrocytes to an environment, which is far from isotonic to evaluate the ability of resealed erythrocytes to withstand the stress and maintain their integrity as well as appearance. Incubating the drug loaded resealed erythrocytes Suspension (10-50% haemotocrit) with distilled water (5ml) for 15 min followed by centrifugation at 2500rpm and the supernatant was estimated spectrophotometrically at 540 nm for percent hemoglobin content.

Stability Studies

All the 6 batches of prednisolone resealed erythrocytes were tested for stability. All the preparation was divided into 3 sets and was stored at 4°C & 45%RH, 37±2°C & 65%RH±5% and at room temperature respectively, in thermostatic humidity control oven (Lab Control Equipment, Mumbai). After 15 days and one-month drug release of the selected formulation (F2) was determined by the method discussed previously in *invitro* drug release studies and percentage drug content studies was also carried out for all the formulations.

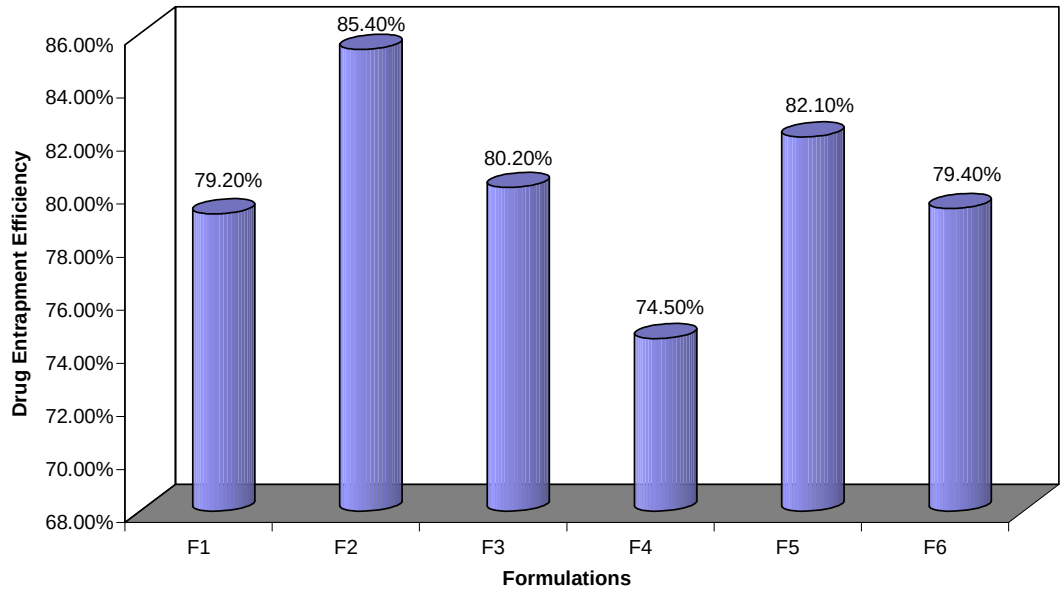
RESULTS AND DISCUSSIONS

I. Physical characterization

Drug content

The drug content was studied in all 6 batches of prednisolone resealed erythrocytes with different cross-linking agents. It was observed that drug to carrier ratio was found to be same with 2 different cross linking agents and drug entrapment efficiency increased with addition of cross linking agents. The maximum entrapment efficiency was found to be in F2 (85.4%) and lowest entrapment in F4 (74.5%) as shown in **Fig No.1**. *In vitro* release studies were based on content of drug present in each formulation.

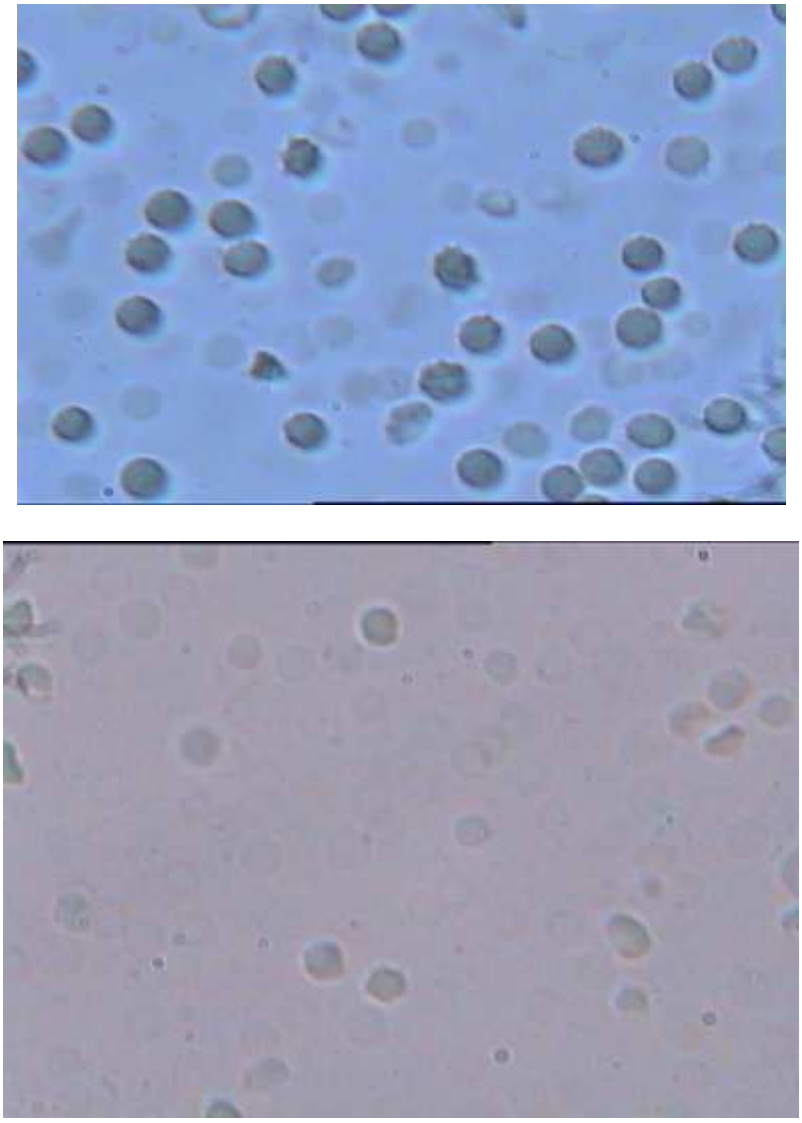
Fig. No.1. Comparison of Drug Entrapment Efficiency between Different Formulations of Prednisolone Resealed Erythrocytes



Shape and surface morphology

Photomicrograph (optical microscopy) of prednisolone-loaded erythrocytes is shown in **Fig No.2.** Different magnifications (45x and 100x) were used while taking these photomicrographs. Average particle size prednisolone resealed erythrocytes was found to be similar to that of normal erythrocytes (7.2µ) respectively.

Figure 2: Photomicrograph of Prednisolone loaded erythrocytes



Drug release

The release profiles of prednisolone from carrier erythrocytes at 37^0C are shown in **Fig No. 3 and 4**. As seen, the efflux of prednisolone from carrier cells at 37^0C follows zero-order kinetics during the experimental period (r2= 0.999). Thus prednisolone can be good candidate for use of carrier erythrocytes as an intravenous slow release system and it is possible to retain the drug in the carrier erythrocytes for prolonged time periods, i.e. entire life span of erythrocytes. It was observed that drug release from the formulations F1, F2 and F3 (Preswell dilution technique) increased as compared to formulations F4, F5 and F6 (dilution technique). This indicates formulations prepared from preswell dilution technique showed higher entrapment efficiency compared to formulations prepared from dilution technique and also F2 showed best-controlled release action. So preswell dilution technique method is more suitable method for preparation of prednisolone resealed erythrocytes.

Fig. No.3. Plots of Cumulative % Drug Released Vs. Time for Formulations F1, F2 & F3 of Prednisolone Resealed Erythrocytes [*In vitro* Release Studies] [Zero Order Plots]

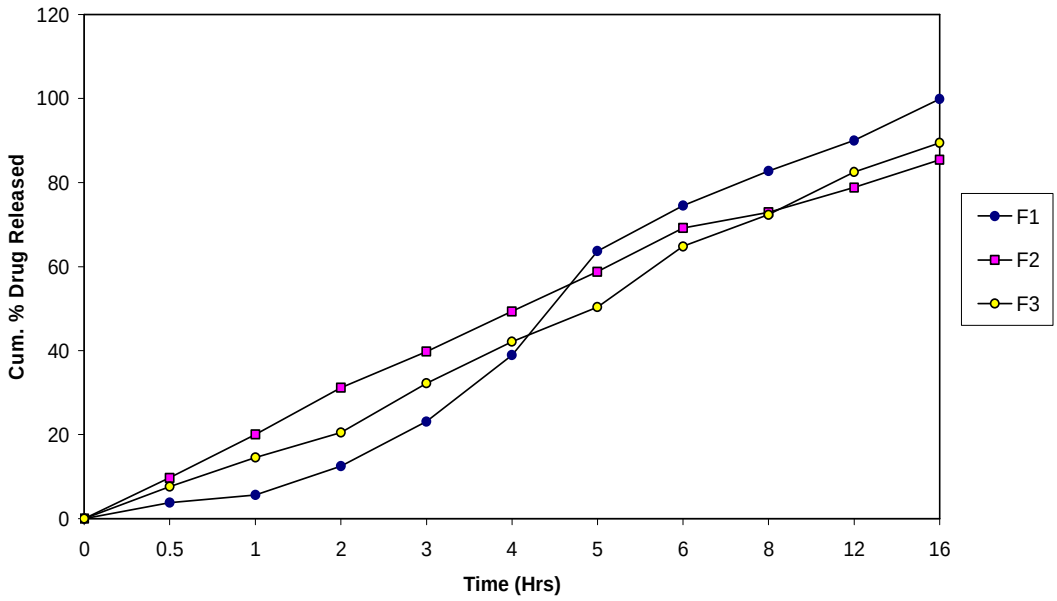
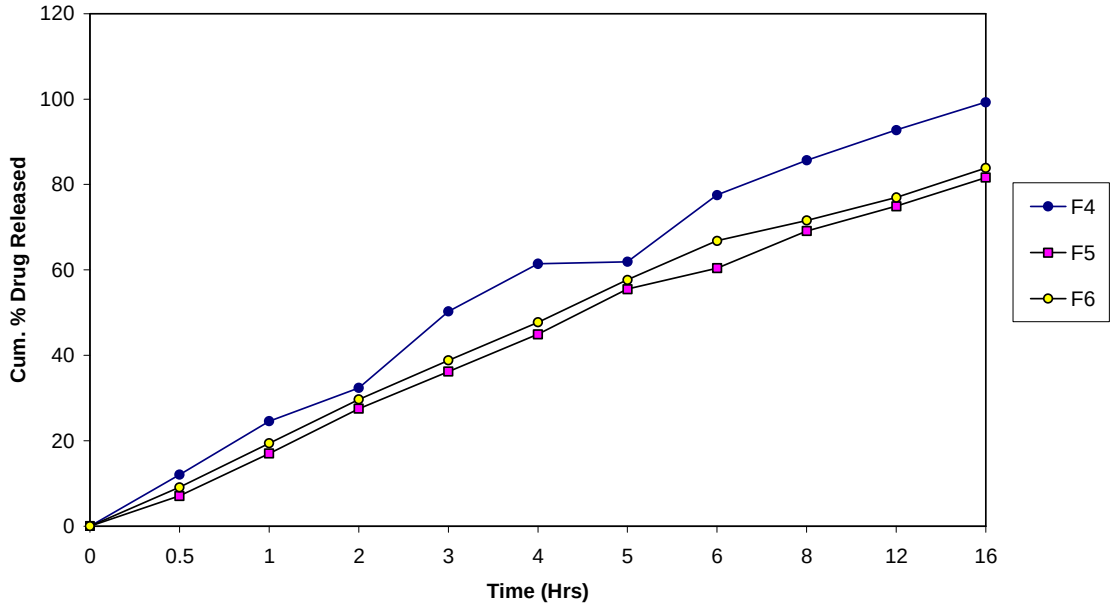


Fig. No.4. Plots of Cumulative % Drug Released Vs. Time for Formulations F4, F5 & F6 of Prednisolone Resealed Erythrocytes [*In vitro* Release Studies] [Zero Order Plots]



II. Cell related characterization

Osmotic Fragility

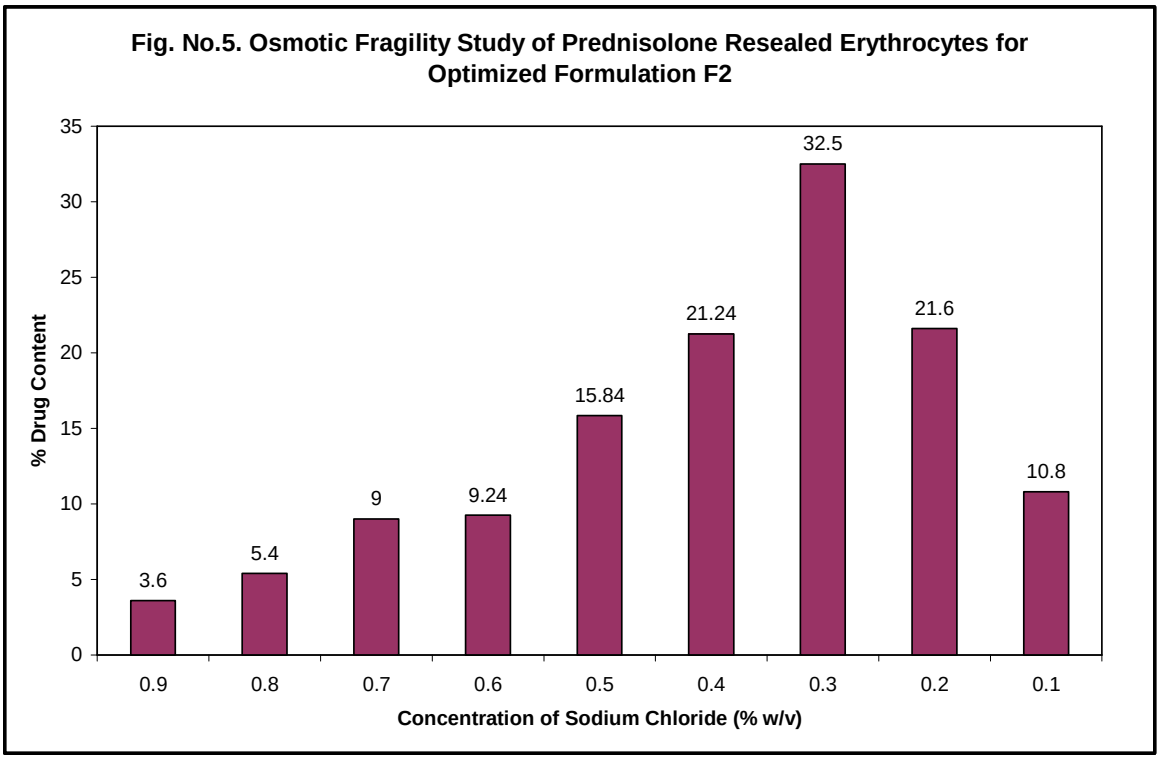
We have developed osmotic lysis method for encapsulation of drug in erythrocytes in this osmolality of the buffer used is crucial. A hypotonic solution of concentration 0.3% (w/v) induced cell swelling and the formation of pores that allowed the drug to penetrate the erythrocyte. However low concentration of sodium chloride indicated greater haemolysis of erythrocytes. The results obtained are depicted in **Table no. 3 and Fig No.5.** It indicates at 0.3%

NaCl there was less resistance of cells to haemolysis as compared to other concentrations of sodium chloride used for the study.

Table No.3 Osmotic Fragility Study Of Prednisolone Resealed Erythrocytes For Optimized Formulation F2

SL NO.	Concentration of Sodium Chloride in %w/v	Absorbance	Concentration in (µg/ml)	Drug content (mg/10mg	% Drug content
1	0.9	0.042	1.0	0.36	3.6
2	0.8	0.087	1.5	0.54	5.4
3	0.7	0.162	2.5	0.9	9.0
4	0.6	0.212	2.65	0.954	9.24
5	0.5	0.321	4.4	1.5	15.84
6	0.4	0.465	5.9	2.1	21.24
7	0.3	0.698	8.4	3.25	32.5
8	0.2	0.484	6.0	2.16	21.6
9	0.1	0.265	3.0	1.08	10.8

Amount of drug taken = 10 mg



Osmotic shock:

Osmotic shock was carried out for the formulation F2 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 formulation was incubated with water for osmotic shock study.

III. Biological characterization

Sterility Test

Formulation was subjected to sterility test in Health Care Diagnostic Centre, Guntur. The results obtained after 7 days of incubation showed no growth of organisms on the culture medium. This indicates that formulation passes the test for sterility.

Stability Studies:
Stability studies of the prepared resealed erythrocytes were carried out by storing all the formulations at 4°C & 45%RH, room temperature and 37°C & 65%RH±5% for one month. Two parameters, percentage drug content and *invitro* release studies of a formulation was carried out. The results revealed that 4°C & 45%RH is the ideal storage condition for prednisolone resealed erythrocytes.

CONCLUSION

Erythrocyte is a suitable carrier for the preparation of prednisolone resealed erythrocytes. Formulation containing glutaraldehyde as cross-linking agent showed maximum drug entrapment efficiency. The photomicrograph analysis revealed that size and shape of drug-loaded erythrocytes was similar to that of normal erythrocytes. The results of or study showed that the carrier erythrocytes having considerable loading parameters, release their drug content with zero order kinetics. Hemoglobin content was found to be satisfactory. Osmotic fragility study indicated 0.3%w/v concentration of sodium chloride (Hypotonic solution) showed maximum drug entrapment and hemoglobin content. Targeting efficiency of drug loaded erythrocytes over free drug is higher, which may provide increased therapeutic index and drug targeting to various organs may help- in the reduction of dose required for the therapy and there by dose related systemic side effects could also be minimized. Present work was a preliminary satisfactory study in designing prednisolone resealed erythrocytes for site specificity and prolonged release of therapy. Further *in vivo* studies need to be carried out and *in vitro-in vivo* correlation needs to be established safety, efficacy and bioavailability of the formulation.

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