ERYTHROCYTES AS CARRIER FOR PREDNISOLONE: *IN VITRO* AND *IN VIVO* EVALUATION

GOPAL V SHAVI[a,b]*, RAJENDRA C DOIJAD[a], PRAFUL B DESHPANDE[a], MANVI FV[a], SREENIVASA REDDY MEKA[b], NAYANABHIRAMA UDUPA[b], OMPRAKASH R[a] AND DHIRENDRA K[a]

[a]Department of Pharmaceutics, KLES's College of Pharmacy, Belgaum-10, Karnataka State, India
[b]Department of Pharmaceutics, Manipal College of Pharmaceutical Sciences, Manipal-576104, Karnataka State, India

ABSTRACT

Resealed erythrocytes, as drug delivery system has tremendous potential to achieve site specificity and prolonged release of drug thereby enhancing therapeutic index and patient compliance. In the present investigation erythrocytes obtained from healthy volunteers were loaded with 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* drug release followed zero-order kinetics, haemoglobin content was found to be satisfactory and osmotic fragility study indicated that increased drug entrapment efficiency was found at 0.3%w/v concentration of sodium chloride (hypotonic solution). *In vivo* tissue distribution studies were carried out for optimized formulation and order of distribution was found to be Liver>Lung>Kidney> Spleen. The developed drug delivery system is endowed with several exclusive advantages and hence holds potential for further research and clinical application.

Keywords: resealed erythrocytes; prednisolone; preswell dilution technique, zero-order release kinetics; tissue distribution; glutaraldehyde; dimethylsulphoxide.

INTRODUCTION

Current pharmaceutical scenario is aimed at development of drug delivery systems with maximum therapeutic benefits for safe and effective management of diseases. The concepts are based on controlled drug delivery, biotechnology and polymer sciences, which surpass all the barriers of diseases. The emerging advances in the development of novel drug delivery technologies are likely to have significant impact on drug industry. Results from researchers have shown for the first time that targeted drug delivery system is possible using erythrocytes. The targeted or site-specific delivery of drugs is indeed a very attractive goal because this provides one of the most potential ways to improve the therapeutic index (TI) of drug whilst minimizing its interaction with non-targeted tissue (Nicholas 1995).

The idea of drug carrier with targeted specificity has fascinated scientists for several years and in the last decade successful efforts have been made to achieve this goal. The ultimate form of targeted drug delivery system should be realization of Paul Ehrlich's "magic bullet" concept (Vyas *et al*., 1996) which documents delivery of drugs exclusively to a pre-selected targeted cell-type.

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 (Eichler *et al*., 1986). Among the methods proposed by investigators for drug loading into intact erythrocytes, three have been studied widely, namely, electrical, preswell dilution and dilution technique for drugs like antineoplastic agents (Lynch *et al*., 1980) and (Mishra *et al*., 2002), angiotensin-converting enzyme inhibitors (Tajerzadeh *et al*., 2001) and (Hamidi *et al*., 2000), systemic corticosteroids (Ogiso *et al*., 1985) and (Rossi *et al*., 2001) and prodrugs (Noel-Hocquet *et al*., 1982).

Prednisolone is a corticosteroid of the class glucocorticoids and is a drug of choice for systemic anti-inflammatory and immunosuppressive effects. It is a

Corresponding author e-mail: gopal_shavi@rediffmail.com

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, India. Sodium chloride (NaCl), potassium dihydrogen orthophosphate, Acetonitrile, glutaraldehyde, dimethyl sulphoxide (DMSO) were laboratory or HPLC grades as required.

Methods

Isolation of Human Erythrocytes

The whole O group blood obtained from registered blood bank (K.L.E.S's Hospital and Medical Research Centre, Belgaum, Karnataka, India) was centrifuged at 3000 rpm for 5 minutes at 4±1°C in a refrigerated centrifuge (Plastocrafts, Bangalore, India). The serum and buffy coats were removed by washing three times with phosphate buffer saline (PBS: NaCl, 150mmol/l; K2HPO4, 5mmol/l; pH 7.4) (Zolla *et al*., 1990). The washed erythrocytes were diluted with PBS and stored at 4 °C until used.

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. 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 (Mishra *et al*.,. 1996).

The suspension was washed three times with phosphate buffer saline(PBS) pH 7.4 and then suitably diluted with PBS and stored at 4°C in refrigerator (F1). By using the above method two more batches of resealed erythrocytes were prepared by using two 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 agents (Jain and Jain, 1997 and Lewis and Alpar, 1984). The whole experiment was carried out by maintaining the temperature between 0-4°C.

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 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 (Vyas and Khar, 2002). The suspension was washed three times with phosphate buffer saline and then suitably diluted with PBS and stored at 4 °C in refrigerator (F4). By using the above method, two more batches of resealed erythrocytes were prepared by using two 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 between 0-4°C.

Table 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

Table 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

Table 3: **Osmotic fragility study of prednisolone resealed erythrocytes for optimized formulation F2.**

S. 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

reconstitution properties were obtained by lyophilizing the formulation.

Procedure for lyophilization

Lyophilization was carried out immediately after the preparation of the suspension. The suspension of drug encapsulated resealed erythrocytes was taken in a 250ml beaker and frozen to a temperature of -40 ^{o}C in the bath compressor of the lyophilizer (Lyodel). The frozen product obtained after 2h in the bath was then transferred to the vacuum trap of the lyophilizer for drying under vacuum and subjected to bath temperature of -40 ^{o}C under a pressure of 10 torr for 4h. After freeze-drying, the lyophilized product was obtained in the form of dry mass (Khar and Diwan, 2001 and Tesconi and Sepassi, 1999). 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 refrigerator until use.

Physicochemical characterization

Drug Assay

Glucocorticoid analogues were determined by HPLC on boiled samples according to the method described by Magnani, *et al.*, 1998. Briefly, 10mg of drug loaded resealed erythrocyte (suspension) from each batch was diluted upto 100 ml with double distilled water, boiled for 5 min and filtered through 0.22μm filter. HPLC determination was 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 was1.0 ml/min and detection was carried out at 239 nm using UV detector with an injection volume of 20μl.

Shape and surface morphology

Particle size and shape analysis was carried out using 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 determine the release kinetics of prednisolone from carrier erythrocytes (lyophilized product) containing 10mg of prednisolone were taken and suspended in 20 ml of isotonic saline, for diffusion in an inverted measuring cylinder using 100 ml of pH 7.4 phosphate buffer as the diffusion medium in a beaker. The opening of the measuring cylinder was covered with cellophane membrane, which acts as a semipermeable membrane. 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 time intervals of 30 min, 1, 2, 3, 4, 6, 7, 8, 12 and 16 h, and replaced by the same volume of PBS pH 7.4 (Gutierrez Millan *et al.*, 2004). These samples were filtered through 0.45μm membrane filter, deproteinized with acetonitrile, diluted suitably and the drug was estimated by using UV spectrophotometer (Shimadzu Corporation, Japan) at 239 nm.

Cell related characterization

Osmotic fragility

To evaluate the resistance of erythrocytes membrane against the osmotic pressure changes in the 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 min, followed by centrifugation at 2500 rpm for 10 min and supernatant was examined for drug content (Jain and Jain 1997). 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 drug loaded resealed erythrocytes suspension was incubated (10-50% haemotocrit) in distilled water (5ml) for 15 min followed by centrifugation at 2500rpm and the supernatent was estimated spectrophotometrically at 540 nm for percent hemoglobin content (Khar and Diwan, 2001).

In vivo tissue distribution studies

The study was carried out for distribution of drug loaded resealed erythrocytes to various organs of reticuloendothelial system like liver, lungs, kidney and spleen. The animal study was approved by Jawaharlal Nehru Medical College animal ethics committee, KLE Campus, Belgaum, Karnataka, India. Nine healthy adult wistar rats weighing 200-240g were selected. A constant day and night cycle was maintained and they were fasted for overnight. The animals were divided into three groups, in which two groups each containing four rats and one group containing one rat. Group I rats received developed resealed erythrocytes equivalent to 900µg of prednisolone intravenously in the tail vein after predispersing them in sterile PBS pH 7.4 solution. Resealed erythrocytes from batch F2 were selected for the study. Group II rats received 900µg of pure prednisolone intravenously. Group III rats was treated as solvent control and injected intravenously with 1.0ml of sterile PBS pH 7.4 solution (Sanz *et al*., 1999 and Pinilla, 1994).

At 1, 2, 4 and 6h, the rats were sacrificed and their liver, lungs, spleen and kidney were isolated. The individual organs of each rat were homogenized separately and digested with 1ml of 70% v/v of acetonitrile for 2h to precipitate the protein. Then the drug was extracted with 25ml of methylene chloride, washed first with 2ml of 0.1N NaOH, followed by 0.1N acetic acid and finally with water. The extract was evaporated to dryness and redissolved in 5 ml of methanol. The drug content was estimated using UV-spectrophotometer at 239nm. Standard amount of prednisolone was taken throughout the study.

Stability studies

All the six batches of prednisolone resealed erythrocytes were tested for stability. All the preparations were divided into three sets and were stored at 5±3°C, 30±2°C/ 65%±5% RH and at room temperature, in thermostatic humidity control oven (Lab Control Equipment, Mumbai, India). After 2 weeks and 1 month, drug release of the optimized formulation (F2) was determined by the method previously discussed and percentage drug content was determined for all the formulations (Carstensen, 1998).

RESULTS AND DISCUSSIONS

Physical characterization

Drug content

The drug content was estimated in all six batches of prednisolone resealed erythrocytes with different cross-linking agents. It was observed that drug to carrier ratio was found to be same with two different cross linking agents and drug entrapment efficiency increased with addition of cross linking agents (Mishra *et al*., 1996) and (Dale *et al*., 1997). The maximum entrapment efficiency was found to be in F2 (85.4%) and lowest entrapment in F4 (74.5%) as shown in fig. 1. *In vitro* release studies and *in vivo* tissue distribution studies were based on content of drug present in each formulation.

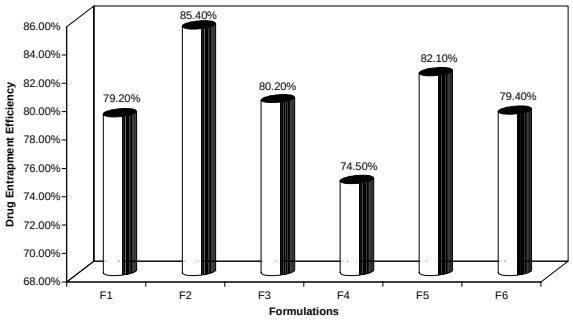

Fig. 1: Comparison of drug entrapment efficiency between different formulations of prednisolone resealed erythrocytes.

Shape and surface morphology

Photomicrograph (optical microscopy) of prednisolone-loaded erythrocytes is shown in fig. 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).

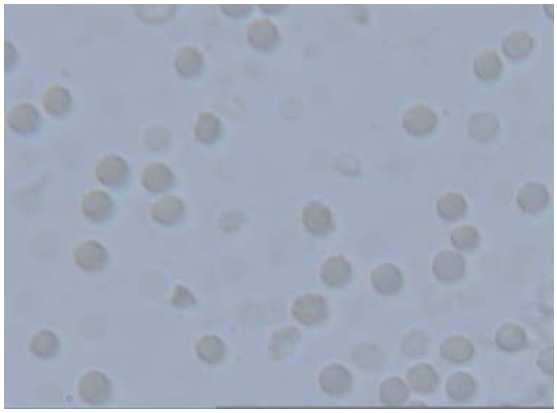

Fig. 2: Photomicrograph of prednisolone resealed erythrocytes

Drug release

The release profiles of prednisolone from carrier erythrocytes at 37°C are shown in fig. 3 and 4. As seen, the efflux of prednisolone from carrier cells at 37 °C follows zero-order kinetics during the experimental period (r^2= 0.999) (Tajerzadeh, 2001). Thus prednisolone can be good candidate for use in 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) was higher when compared to formulations F4, F5 and F6 (dilution technique). This indicates that formulations prepared from preswell dilution technique showed higher entrapment efficiency compared to formulations prepared from dilution technique. Also, F2 showed controlled release profile. Hence preswell dilution technique method is more suitable for preparation of prednisolone resealed erythrocytes (Dhir and Bhaskaran, 1995).

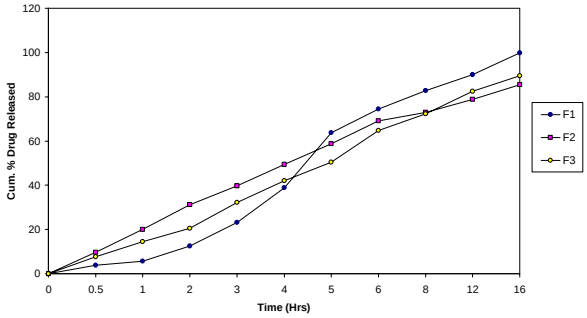

Fig. 3: *In vitro* release profiles of formulations F1, F2 and F3.

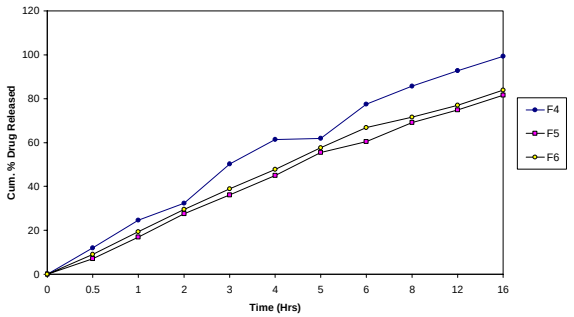

Fig. 4: *In vitro* release profiles of formulations F4, F5 and F6.

Cell related characterization

Osmotic fragility

In the developed osmotic lysis method for encapsulation of prednisolone, 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 haemolysis of erythrocytes was greater with low concentration of sodium chloride. (Richieri, 1985). The results obtained are depicted in table 3. It indicates that at 0.3% NaCl there was less resistance of cells to haemolysis as compared to other concentrations of sodium chloride used for the study.

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 haemoglobin from the cell (Murthy *et al*., 2001). This indicates that there was complete lysis of the erythrocytes when formulation was incubated with water for osmotic shock study.

In vivo tissue distribution studies

Optimized formulation F2, with highest drug content was selected for *in vivo* tissue distribution studies. The comparison between the amount of drug distributed from resealed erythrocytes and free drug in various organs is presented in fig. 5 and 6.

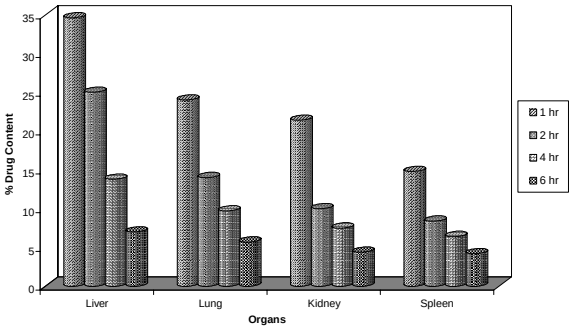

Fig. 5: *In vivo* tissue distribution studies of pure prednisolone.

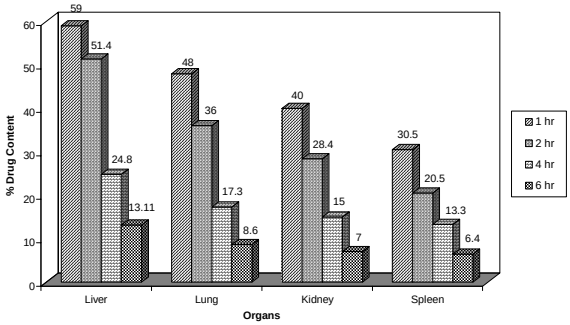

Fig. 6: *In vivo* tissue distribution studies of optimized formulation F2.

These results revealed that the drug loaded erythrocytes showed preferential drug targeting to liver followed by lungs, kidney and spleen (Sanz, 1999). It was also revealed that as compared to pure drug, higher concentration of drug was distributed to the organs after administering the dose in the form of resealed erythrocytes and the order of drug distribution was found

to liver>lungs>kidney>spleen. Large amounts of distribution in liver may be attributed to uptake of the drug loaded erythrocytes by reticuloendothelial system and large size of liver as compared to other organs (Wadke, 1980).

Stability studies

Stability studies of the prepared resealed erythrocytes were carried out by storing all the formulations at 5±3°C, 30±2°C/65%±5% RH and at room temperature for 2 weeks and 1 month period (Carstensen, 1998). Parameters like percentage drug content and *in vitro* release studies of the formulation were carried out. The results revealed that 5±3°C 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 present 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 using 0.3% w/v concentration of sodium chloride (Hypotonic solution) showed maximum drug entrapment and hemoglobin content. *In vivo* tissue distribution studies indicate that order of tissue distribution was Liver>Lung>Kidney> Spleen. Targeting efficiency of drug loaded erythrocytes over free drug is higher, which may provide increased therapeutic index and drug targeting to various organs. It 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 in designing prednisolone resealed erythrocytes for site specificity and prolonged release of therapy. Further elaborate *in vivo* studies need to be carried out and *in vitro-in vivo* correlation needs to be established for the safety, efficacy and bioavailability of the formulation.

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