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ERYTHROCYTES AS CARRIERS OF METRONIDAZOLE : IN-VITRO CHARACTERIZATION

Naresh Talwar and N.K.Jain*
Department of Pharmaceutical Sciences
Doctor Harisingh Gour Vishwavidyalaya,
(Formerly University of Saugar)
SAGAR (M.P.) 470 003 INDIA

ABSTRACT

Rat erythrocytes were loaded with metronidazole by a method based on hypotonic preswelling, hemolysis, isotonic resealing and reannealing. The encapsulation efficiency of 42-56% was achieved. The loaded cells exhibited elevated osmotic fragility and lower resistance to turbulence shock as compared to the normal cells and were found to release encapsulated drug slowly. The glutaraldehyde treatment of the cells resulted in the stabilization of loaded cells, which were found to be highly resistant to the osmotic and turbulence shocks. In-vitro release of metronidazole was also retarded upon treatment, and was dependent upon the concentration of glutaraldehyde. The loaded erythrocytes were obtained in powder form, ready for reconstitution, with a view to improve the shelf life. On the basis of in-vitro studies glutaraldehyde treated erythrocytes appeared to be promising carriers of metronidazole.

* To whom correspondence should be directed.

INTRODUCTION

Metronidazole is a nitro-imidazole which is used as an antiprotozoal and antimicrobial agent for many years. It is the primary drug used in the treatment of extra-intestinal involvement of amoeba (hepatic abscess) (1).

Labhasetwar and Dorle (2) have reported therapeutic inefficacy of metronidazole in the treatment of extra-intestinal (hepatic) amoebiasis, which they ascribed to the ineffective drug concentrations in the liver. They have proposed and characterized in-vitro a nanoparticle based carrier system for selective localization of drug to the liver.

Erythrocytes have been recognized as biodegradable and biocompatible carriers for a variety of bioactive agents. Low molecular weight drugs and high molecular weight proteins can be encapsulated in erythrocytes by reversible hypotonic lysis (3), electrical breakdown (4), chemicals mediated membrane perturbation (5) or endocytosis (6). Drug carrier erythrocytes can be utilized for targeting of encapsulated agents to the reticulo-endothelial system (7), to provide sustained release of encapsulated agents in circulation (8) or to function as circulating bio-reactors especially for enzymes (9). Erythrocytes offer various advantages over other drug carriers with respect to biocompatibility, which can be optimised by the use of autologous erythrocytes and large amounts of material can be encapsulated in relatively small volume of cells (3).

In the present report we propose and in-vitro characterize erythrocytes as carriers of metronidazole for its selective localization to the liver in the treatment of hepatic amoebiasis. The liver localization of drug is anticipated to improve its therapeutic performance and may result in

minimization of toxic manifestations by reducing the exposure of drug to non-target tissues.

MATERIALS AND METHODS

Materials

Metronidazole (Unique Labs, Bombay, India) and Glutaraldehyde (25% aqueous solution, Fluka, Switzerland) were used. All other chemicals were of AR grade. Solvents for HPLC were of Lichrosolv grade (E.Merck, India).

Methods

1. Isolation of erythrocytes from blood

Blood was drawn from male albino rats (weighing around 250 g) by cardiac puncture and was anticoagulated with heparin. Freshly collected blood was centrifuged at 2000g for 5 min in a refrigerated centrifuge (Eltek, SICO, India) at 4°C. The plasma and the buffy coat were carefully pipetted off and thus obtained packed erythrocytes were washed thrice with phosphate buffered saline (PBS; NaCl, 150m mol/l, K_2HPO_4/KH_2PO_4, 5m mol/l, pH 7.4). The erythrocytes obtained after third wash were mixed with sufficient PBS to obtain a hematocrit of 50%.

2. Encapsulation of metronidazole in erythrocytes

Metronidazole was encapsulated in erythrocytes by following the 'preswell loading procedure' described by Field et al. (10), with minor modification. The erythrocyte suspension (2 ml) was centrifuged at 2000g for 5 min at 4°C to obtain 1 ml of packed cells. To the packed cells, 4 ml of 0.65% sodium chloride solution was added and the contents were gently mixed. The suspension was centrifuged at 600g for 5 min to recover the swollen cells and the supernatant was discarded. The cell hemolysate (100 µL; prepared by

lysing packed erythrocytes with water, 1:1) was layered over the top of the cells and metronidazole solution (5 mg/ml in water) was added in 100 µL portions, until the cells reached the point of lysis. Three hundred microlitres (300 µL) of drug solution was found to be sufficient to bring the cells to the point of lysis, further addition resulted in complete lysis of the cells. Calculated amount of hypertonic saline was added to restore the isotonicity. The suspension was incubated at 37°C for 15 min to allow annealing of the cells, following which the cells were washed thrice with PBS to remove the released hemoglobin and the unentrapped drug. The cells were finally suspended in PBS.

3. Determination of drug entrapment

A high performance liquid chromatographic (HPLC) method developed by the authors (11) was employed for the estimation of metronidazole. The packed erythrocytes (0.1 ml) obtained after loading were lysed by diluting with distilled water (2 ml) and methanol (5 ml) was added for deproteinization. The suspension was kept in dark for 1h and was centrifuged at 3000g for 10 min. The clear supernatant was withdrawn and filtered through 0.45 µ filter (Whatman). Samples (20 µL) were injected in the HPLC apparatus. The chromatographic conditions were as reported (11).

4. Glutaraldehyde treatment of loaded cells

Loaded cells were stabilized by treatment with glutaraldehyde. The effect of glutaraldehyde concentration upon various parameters of loaded cells was investigated. Aliquots (1 ml, 50% hematocrit) of drug loaded cells were incubated with 5 volumes of 0.1, 0.3 and 1% solutions of glutaraldehyde (v/v in PBS) at 37°C for 10 min. After incubation, the suspension was diluted with 25 volumes of PBS to stop the reaction, and the cells were washed thrice with PBS.

5. In-vitro characterization of loaded erythrocytes

a. Metronidazole and hemoglobin release

In-vitro leakage of drug and hemoglobin were monitored from drug loaded and drug-loaded glutaraldehyde treated cells. The cell suspensions (5% hematocrit in PBS, 5 ml) were stored at 4°C in amber colored glass bottles. Periodically the clear supernatants were withdrawn, deproteinized using methanol and were estimated for metronidazole content by HPLC, as described earlier. The hemoglobin leakage was monitored by recording the absorbance of the supernatant at 540 nm on a spectrophotometer (Shimadzu UV-150-02, Japan). The percent hemolysis was determined by comparison of absorbance of supernatant with the absorbance obtained after hemolysis of same number of cells in distilled water (12).

b. Osmotic fragility studies

Osmotic fragility study was performed for normal and drug loaded erythrocytes following the method described by Sprandel and Zollner (13). Erythrocytes (0.5 ml packed cells) were incubated in sodium chloride solutions of different strengths (0.9, 0.85, 0.8 ..... 0.1 g%) for 10 min at 37°C. The suspensions were centrifuged and the supernatants were estimated for hemoglobin leakage spectrophotometrically.

c. Osmotic and turbulence shock study

For osmotic shock study erythrocyte suspensions (1 ml, 10% hematocrit) were diluted in distilled water (5 ml) and centrifuged at 2500g for 5 min. The supernatant was estimated for hemoglobin content.

Turbulence shock study was performed as described by DeLoach et al (14). The erythrocyte suspension (5 ml, 10% hematocrit) was passed through a 22 gauge needle several

times at a flow rate of 10 ml/min and release of hemoglobin after different numbers of passings was determined.

d. Morphological examination and cell counting

The morphological examination of cells was performed on a light microscope (Leitz, Biomed, Germany). The cell counting was performed on a hemocytometer.

6. Drying of glutaraldehyde treated erythrocytes

The erythrocytes (0.3% glutaraldehyde treated) were collected on a sintered glass funnel (G-4, Borosil, India) by filteration and dried in vacuum (at 200 mm of Hg) for 10h. Alternatively the erythrocyte suspension was filled in vials and lyophilized at -40°C to 0.01 torr using a laboratory lyophilizer (SICO, India). The dried powder was filled in amber colored glass vials and stored at 4°C for a month.

RESULTS AND DISCUSSION

A method based on hypotonic preswelling, hemolysis, isotonic resealing and reannealing was employed for the encapsulation of metronidazole in erythrocytes. Swollen erythrocytes were brought to the point of lysis by 300 µL of aqueous drug solution. The point of lysis was observed conveniently in the manner described by Lewis and Alpar (15). A thin layer of white ghosts was observed on the top of the packed cells. Various encapsulation parameters are recorded in Table-1. The encapsulation of 0.71 ± 0.095 mg of metronidazole per millilitre of packed erythrocytes was recorded which represented 42-56% efficiency of encapsulation. Satisfactory cell recoveries were obtained.

The light microscopic examination of the drug loaded cells revealed no appreciable difference in cell morphology as compared to the normal cells. The loaded cells were found of

TABLE 1

Encapsulation Parameters

Parameter	Value (mean ± S.D.; n=4)
Metronidazole encapsulation*	0.71 ± 0.095 mg
Percent cell recovery	90 ± 3.78
Percent cellular hemoglobin	75.6 ± 4.61

* Encapsulation per millilitre of packed erythrocytes.

biconcave-discoid shape like the normal cells. Few of the loaded cells (~2%) exhibited spherocytic transformation.

Metronidazole loaded erythrocytes were characterized for various parameters viz. drug and hemoglobin release, osmotic fragility, turbulence shock and osmotic shock to get an insight into stability of the carrier erythrocytes. The various characterized parameters are recorded in Table-2.

The osmotic fragility curves of the normal and drug-loaded erythrocytes are shown in Fig.-1. The normal erythrocytes released 50% of cellular hemoglobin at sodium chloride concentration of 0.38g%, whereas the drug loaded erythrocytes released the same at elevated salt concentration i.e. 0.53g%. The drug loaded erythrocytes exhibited deviation from the osmotic fragility curve of the normal erythrocytes. The loaded cells released higher amounts of hemoglobin at all concentrations. The loaded erythrocytes, thus exhibited higher osmotic fragility as compared to normal cells. Such cells of elevated fragility may be destroyed rapidly in circulation and may result in reduced shelf life (13).

TABLE 2

Characterization of normal, drug loaded and drug loaded glutaraldehyde treated erythrocytes

Parameter	Normal cells	Drug loaded cells	Drug loaded treated cells
Shape	Biconcave discs	Biconcave discs	Biconcave discs
Osmotic fragility*	0.38 ± 0.02g%	0.53 ± 0.017%	-
Turbulence fragility**	20	13	-

* Sodium chloride concentration producing 50% hemoglobin loss.

** Number of passes through a 22 gauge needle producing 50% hemoglobin loss.
Values represented as mean ± S.D. (n=4).

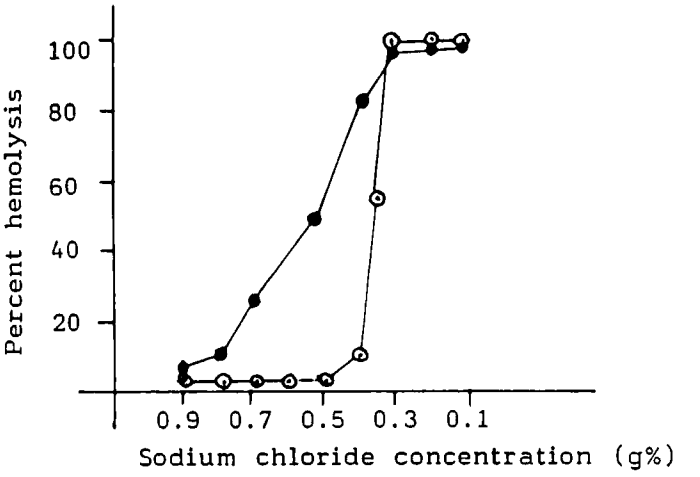

FIGURE 1

Osmotic fragility curves of normal (0) and metronidazole loaded (●) erythrocytes.

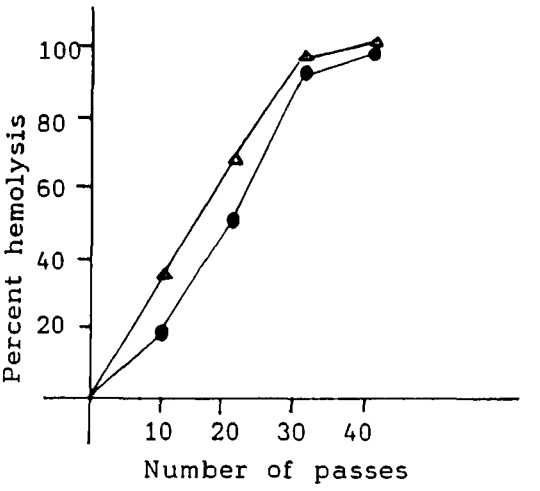

FIGURE 2

Turbulence shock study of normal (●) and metronidazole loaded (△) erythrocytes

Turbulence-shock study of the loaded cells was performed to evaluate the stability of the loaded cells against the turbulence stress exerted by the cells upon shaking of drug loaded cells in-vitro and against in-vivo circulation turbulence. The cells were subjected to turbulent flow by passing the suspension several times through a 22 gauge needle. The normal erythrocytes released 50% of cellular hemoglobin upon 20 passes, whereas the drug loaded erythrocytes took 13 passes to release the same amount (Fig.- 2). The drug-loaded cells thus appeared less resistant to the turbulence shock, indicating that the destruction of cells may result on shaking the sedimented cells to redisperse them and cell destruction may also take place during circulation.

In-vitro release profiles of metronidazole and hemoglobin from loaded cells are shown in Fig.-3. Slow release of the

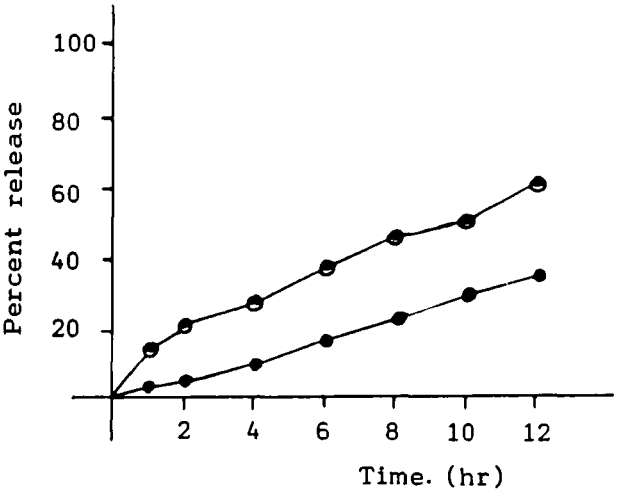

FIGURE 3

In-vitro release of metronidazole (⊖) and hemoglobin (●) from drug-loaded erythrocytes

drug was recorded. In 12h, 60% of the encapsulated drug was found in the extracellular media. Similarly 34% of hemoglobin was found extracellular.

The drug loaded cells thus exhibited poor stability as evident from the osmotic fragility, turbulence shock study and in-vitro release profiles. Glutaraldehyde treatment of loaded cells facilitated stabilization. The osmotic shock study revelaed that there was negligible to nil hemoglobin loss when drug-loaded glutaraldehyde treated cells were added to water (hypotonic), in contrast to the non-treated cells, which, exhibited complete lysis. The glutaraldehyde treated cells thus exhibited complete resistance to the osmotic stock.

The turbulence shock study of the glutaraldehyde treated cells (0.1, 0.3 and 1% treated) exhibited the release of 5-12%

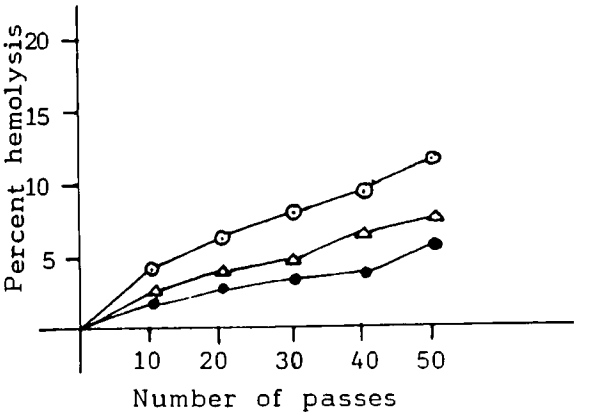

FIGURE 4

Turbulence shock study of glutaraldehyde treated erythrocytes (⊙) 0.1%, (△) 0.3%, (●) 1% treatment

of cellular hemoglobin upon 50 passes (Fig.-4). The cells treated with higher concentrations of glutaraldehyde appeared to be more resistant.

In-vitro release of metronidazole from glutaraldehyde treated cells is shown in Fig.-5. Glutaraldehyde treatment resulted in reduction in efflux rate of the drug. The rate of release of metronidazole was decreased as the concentration of glutaraldehyde used for treatment increased. Glutaraldehyde treatment of erythrocytes results in crosslinking of the membrane proteins (14). The observation of decrease of drug efflux upon increasing the glutaraldehyde concentration can be ascribed to the degree of crosslinking, which increases upon exposure of cells to higher concentrations of glutaraldehyde. The 0.1, 0.3 and 1% treated cells exhibited the release of 32%, 20% and 9% respectively of encapsulated drug in 12 h. Negligible to nil efflux of hemoglobin was recorded from treated cells.

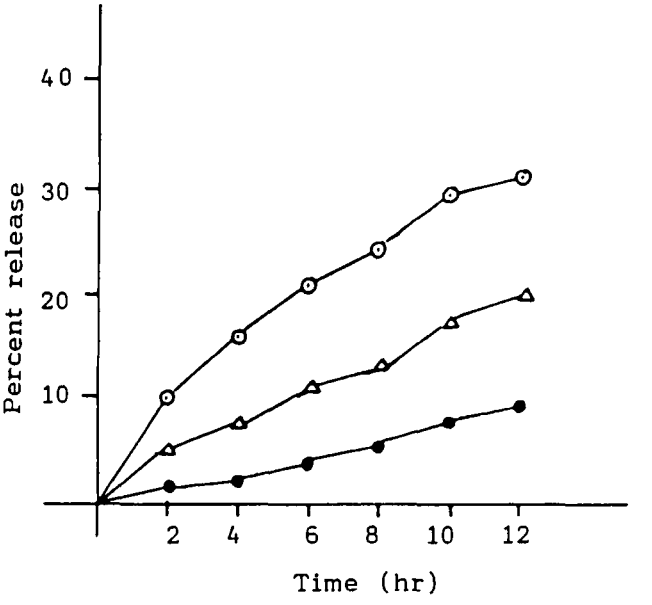

FIGURE 5

In-vitro release of metronidazole from glutaraldehyde treated erythrocytes (●) 1%, (△) 0.3% and (○) 0.1% treatment.

The glutaraldehyde treatment of the drug loaded cells thus resulted in improved stability of the carrier erythrocytes. The glutaraldehyde treatment of erythrocytes is also reported to target them to the reticulo-endothelial system (RES) (7). The treated cells are cleared from circulation rapidly by liver and spleen recognition. Thus the administration of glutaraldehyde treated metronidazole loaded erythrocytes is anticipated to result in rapid uptake of the drug by the liver tissue. The elevated concentration of drug in the liver is anticipated to improve the therapeutic efficacy of metronidazole.

Maximum shelf life of 2 weeks has been reported for drug carrier, non-glutaraldehyde treated erythrocytes (15).

With a view to improve shelf life of the carrier, an attempt was made to obtain loaded cells in powder form, ready for reconstitution. The glutaraldehyde treated cells were easily collected on a sintered glass funnel and dried to obtain a powder. The cells were also obtained in powder form by lyophilization. The powdered cells upon light microscopic examination exhibited no change in morphology of the cells, as compared to the normal cells. Some cell aggregates were however found, which were easily redispersed by passing the suspension through a 26 gauge needle. The powdered cells were stored at $4^{\circ}C$ for a month in amber coloured glass container, following which in-vitro release of the drug was investigated. No appreciable change in in-vitro release profile of metronidazole was detected. Further extensive studies are required to investigate the bio-chemical changes that may take place in cells upon storage to account for their suitability as carriers of metronidazole.

The glutaraldehyde treated erythrocytes are reported to be well tolerated by the liver, no damage results from their extensive and prolonged uptake (7). On the basis of in-vitro studies, glutaraldehyde treated erythrocytes thus appeared as promising carriers of metronidazole. The shelf life of the loaded erythrocytes may be improved by storing then in powder form.

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REFERENCES

1. B.E.Roche and B.L.Kier, in "Principles of Medicinal Chemistry", W.O. Foye, ed, 1981, p. 801.
2. V.D. Labhasetwar and A.K.Dorle, J.Controlled Release, 12, 113 (1990).
3. G.M. Ihler, Pharmac.Therap. 20, 151 (1983).
4. U.Zimmermann, F.Riemann and G.Pilwat, Biochim.Biophys Acta., 436, 460 (1976).
5. M.M. Billah, J.B. Finean, R.Coleman and R.H.Michell, Biochim.Biophys.Acta., 433, 54 (1976).
6. N.Talwar and N.K. Jain, J.Microencapsulation (in press).
7. E.Zocchi, M.Tonetti, C.Polvani, L.Guida, V.Benatti and A.DeFlora, Biotechnol Appl.Biochem., 10, 555 (1988).
8. J.R.DeLoach and C.Barton, Am.J.Vet.Res. 43, 2210 (1982).
9. M.Magnani, M.Laguerre, L.Rossi, M.Bianchi, P.Ninfali, F.Mangani and C.Ropars, Alcoholism : Clin.Exp.Res., 13, 849 (1989).
10. W.N. Field, M.D. Gamble and D.A. Lewis, Int.J.Pharm., 51, 175 (1989).
11. N.Talwar and N.K. Jain, Indian Drugs (in press).
12. K.P.Mishra and B.B.Singh, Ind.J.Exp.Biol., 24, 737, (1986).
13. U.Sprandel and N.Zollner, Res.Exp.Med., 185, 77, (1985).
14. J.DeLoach, S.Peters, O.Pinkard, R.Glew and G.Ihler, Biochim et Biophys.Acta, 496, 507 (1977).
15. D.A.Lewis and H.O.Alpar, Int.J.Pharm., 22, 137 (1984).