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RESEARCH PAPER

Evaluation of Hypotonic Preswelling Method for Encapsulation of Enalaprilat in Intact Human Erythrocytes

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ABSTRACT

The hypotonic preswelling method for encapsulation of drugs in intact human erythrocytes was evaluated using enalaprilat as a model peptidelike drug. Several process variables, including volume, concentration, pH, and method of addition of drug solution, type of erythrocyte-suspending medium, temperature, initial packed density of erythrocytes, and individual process steps, were exploited with respect to their effects on the loading parameters (i.e., loaded amount, efficiency of entrapment, and cell recovery). In addition, the probable mechanism by which the erythrocytes were loaded by enalaprilat at the point of lysis was shown to be a simple concentration gradient–based diffusion through membrane openings occurring on hemolysis. Finally, the adopted method was validated, and the results showed a considerable degree of reproducibility and recovery for the entire loading procedure.

Key Words: *Enalaprilat; Erythrocyte; Erythrocyte-based drug delivery; Human erythrocytes; Hypotonic preswelling method.*

INTRODUCTION

Enalaprilat, being the pharmacologically active metabolite of the widely used angiotensin-converting enzyme inhibitor (ACEI) enalapril, is also available as intravenous injectable formulations, for management of both hypertension and congestive heart failure (CHF) (1).

Erythrocytes, readily available and abundant biological microspheres, have been exploited for their potential applications as drug delivery carriers (2–6). The biocompatibility and considerable life span of the autologous carrier erythrocytes allow them to serve as prolonged-release intravenous reservoirs of drugs and enzymes (7–12). In addition, the possibility of targeting the bioactive

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agents to the reticuloendothelial system (RES) using the erythrocyte carriers has been reported (13–16). Among the methods proposed by investigators for drug loading of intact erythrocytes (3), three are studied widely: electrical methods (4,17–19), hypotonic dialysis (20–23), and hypotonic preswelling (9,11,12,14,24,25). In the present study, the hypotonic preswelling procedure was evaluated extensively with respect to several process variables using enalaprilat as a model drug. Furthermore, the mechanism of drug loading in human erythrocytes during the encapsulation process was studied; finally, the process validation analysis was performed on the loading method.

EXPERIMENTAL

Materials

Enalaprilat (Pharmhispania S. A. Pharmaceuticals, Spain) was a gift of Dr. Abidi Company (Tehran, Iran). Other chemicals and solvents were prepared locally and were laboratory or lichrosolv grade as needed.

Preparation of Human Erythrocytes

Blood samples were withdrawn by venipuncture from healthy volunteers aged 28 to 35 years using commercially available citrated vacuumed test tubes (Vacutainer, Becton Dickinson, Rutherford, NJ). After centrifuging at 600*g* for 5 min, the plasma and buffy coat were separated, and the remaining packed erythrocytes were washed three times with eutonic modified K-reversed Hank's balanced salt solution (HBSS), which consists of 10.18 g/L KCl, 0.1g/L KH_2PO_4, 1.273 g/L $NaHCO_3$, 0.316 g/L NaCl, 0.08 g/L Na_2HPO_4, and 2.0 g/L glucose. The pH of the solution was adjusted to 7.4 using H_3PO_4 (85% w/v).

Encapsulation of Enalaprilat in Human Erythrocytes

A hypotonic preswelling method described by Pitt et al. (24) was used for loading the human erythrocytes by enalaprilat. For this purpose, 1 ml of washed packed erythrocytes was transferred gently to a silicone-treated test tube (Monoject, Sherwood Medical, Ballymoney, N. Ireland), and 4 ml of a hypotonic modified HBSS with an osmolarity of 0.67 (that of a eutonic solution) was added. The resulting suspension was mixed thoroughly by several gentle inversions and was centrifuged at 600*g* for 10 min. The supernatant was discarded, and the remaining swollen cells were layered by the addition of 200 μl of hemolysate prepared by diluting another portion of erythrocytes with distilled water (1:1). This hemolysate layer presumably serves as an osmotic shock barrier and also as a reservoir of cell constituents for underlaying cells. Then, an aliquot of 250 μl of aqueous solution of enalaprilat (8 mg/ml) was gently added to the cell suspension, and the mixture was inverted gently several times and centrifuged at 600*g* for 5 min. Addition of drug solution and centrifuging were repeated successively two more times to achieve lysis of the cells. This point was detectable by a sudden increase in transparency of the cell suspension and the disappearance of a distinct boundary between cells and supernatant on centrifuging. At this point, the erythrocytes were resealed by the rapid addition of 100 μl of hypertonic modified HBSS, followed by gentle mixing of the suspension. Finally, the resulting mixture was incubated at 37°C for 30 min to reanneal the resealed cells. The carrier erythrocytes obtained by this manner were washed three times using 10 ml of eutonic modified HBSS to wash out the unentrapped drug and the released hemoglobin during the process.

Drug Assay

A reversed-phase high-performance liquid chromatographic (HPLC) method using a mixture of acetonitrile, water, and H_3PO_4 (90:10:0.85) as the mobile phase, a pH-resistant C_{18} column (Shodex Rspak, D18-613, Showa Denko k.k., Tokyo, Japan) as the stationary phase, and an ultraviolet detector with a wavelength of 215 nm was developed for determination of enalaprilat concentration (26). To determine the amount of loaded drug, 0.1 ml of carrier erythrocytes was diluted with 2 ml of distilled water to complete cell lysis, and 2 ml of methanol was added to the resulting lysate for precipitation of cell proteins. Then, the test tube was kept at room temperature for 30 min, followed by centrifuging of the suspension at 3000*g* for 20 min. Finally, 50 μl of the supernatant was injected in the chromatograph.

Loading Parameters

To evaluate the effect of any changes in encapsulation method variables on the loading efficiency, three indices were defined as loading parameters for use throughout the present study: (*a*) loaded amount, the amount of enalaprilat encapsulated in 0.1 ml of the final packed erythrocytes; (*b*) efficiency of entrapment, the percentage ratio of the loaded amount of enalaprilat to the amount added

per 0.1 ml of initial packed cells during the entire loading process; (*c*) cell recovery, the percentage ratio of the hematocrit value of initial packed cells to that of the final loaded cells measured using equal volumes of total suspensions.

Methodological Tests

Incubation of Intact Erythrocytes with Isotonic Drug Solution

To investigate the uptake of enalaprilat by human erythrocytes irrespective of loading condition, 1 ml of washed packed erythrocytes was incubated at 37°C with 9 ml of drug solutions in eutonic modified HBSS with concentrations of 1, 8, 80, and 800 μg/ml. At 0, 30, and 60 min, 0.5 ml of cell suspension was withdrawn and centrifuged at 600*g* for 5 min, and the concentration of enalaprilat in the supernatant was determined by direct injection to the chromatograph. In addition, the concentration of drug in the cellular fraction was also determined at the end of the incubation period after lysis and deproteinization as described above.

Erythrocyte-Suspending Medium

Two types of cell-suspending solutions frequently used during encapsulation of different drugs and other bioactive agents in intact erythrocytes are modified K-reversed HBSS (see above) and phosphate buffered saline (PBS; 150 mmol/L NaCl; 5 mmol/L K_2HPO_4; pH 7.4). The entire loading procedure was performed using each of the solutions, and the loading parameters were determined in each case.

Volume of Drug Solution

To verify the observed point of lysis and to optimize the volume of drug solution used during the loading procedure, the process was performed on six separate erythrocyte samples so that, to each of the cell suspensions, 4, 5, 6, 7, 8, and 9 aliquots of l00 μl of drug solution were added; after completion of the procedure, the loading parameters were determined in each case.

Method of Addition of Drug Solution

A total volume of 700 μl of drug solution (selected as the optimum volume according to the results discussed in the previous section) was added four ways: (*a*) as seven successive portions of 100 μl; (*b*) as three successive 230-μl portions; (*c*) as two successive 350-μl portions of; and (*d*) as one 700-μl portion. After completion of the process, the loading parameters were determined in each case.

Concentration of Drug Solution

The encapsulation procedure was performed using aqueous solutions of enalaprilat with concentrations of 1, 2, 4, 6, and 8 mg/ml, and the loading parameters were determined in each case.

pH of Drug Solution

Two distinct experimental runs, one using unchanged drug solution (pH 2.8) and the other with drug solution with a pH of 7.4 (adjusted by NaOH concentrated solution), were performed, and the results were compared with respect to loading parameters.

Centrifugation Temperature

Two experiments, one with the centrifugation steps at room temperature and the other at 4°C, were carried out, and the corresponding loading parameters were determined.

Packed Density of Erythrocytes

The exact volume of 4 ml of the washed packed erythrocytes were suspended in an equal volume of eutonic modified HBSS; after gentle mixing, the suspension was centrifuged at 600*g* for 5 min. The supernatant was discarded, and the remaining packed cells were divided into four 1-ml aliquots by taking samples from the surface of the cell layer. The cell samples obtained in this manner had different packed densities. Then, each 1-ml portion was subjected to the encapsulation procedure; finally, the loading parameters of the resulting carrier cells were determined.

Variations in Process Steps

A series of experiments was performed as follows: (*a*) without the preswelling step, (*b*) without the lysate layer addition step, (*c*) without both the preswelling and lysate layer addition steps, (*d*) without the reannealing step, (*e*) with the lysate layer prepared by drug aqueous solution instead of water, and (*f*) with the lysate layer prepared by neutralized drug aqueous solution instead of water. Then, the loading parameters were determined for each of the cases.

Mechanism of Entrapment

To investigate the possible mechanism of entrapment, the encapsulation procedure was performed, and the concentration of drug in each of three final washing solutions was determined by HPLC. Then, the total amount of washed out (unentrapped) drug was calculated by considering the total volume of discarded solution. On the other hand, the total amount of entrapped drug was determined using the loaded amount multiplied by the cell recovery of the method (see Loading Parameters section). Finally, taking the volume fraction of cells at the point of lysis, the mechanistic behavior of erythrocytes against the drug molecules was exploited.

Process Validation Tests

The following tests were carried out to validate the whole encapsulation process: within- and between-run variations, intersubject variations, and recovery.

Within- and Between-Run Variations

To evaluate within- and between-run variations, six samples of erythrocytes obtained from a healthy volunteer were subjected to the loading procedure during a single and six different experimental runs, respectively, and the loading parameters and the corresponding coefficients of variation (CV%) were determined.

Intersubject Variations

Blood samples were collected from four healthy volunteers (two male and two female subjects), and the loading procedure was carried out in each case. The loading parameters for each of the subjects as well as the corresponding coefficients of variation were determined.

Recovery

The measured entrapped, unentrapped, and total amount of enalaprilat recovered after completion of the encapsulation procedure were compared with those calculated by considering the total added amount and the volume fractions of cells and supernatant at the point of lysis; the results were expressed as percentages.

RESULTS AND DISCUSSION

Enalaprilat Uptake by Erythrocytes

The results of incubation of intact human erythrocytes with enalaprilat isotonic solutions with different concentrations are shown in Table 1. As can be seen, regardless of the concentration added, no significant amount of drug was taken up by intact erythrocytes. Therefore, it can be said that the erythrocyte membrane has no active role in the encapsulation of enalaprilat in human erythrocytes.

Erythrocyte-Suspending Medium

The loading parameters obtained using the two widely used suspending media (i.e., modified HBSS and PBS) are shown in Table 2. From the data, it can be seen that the modified HBSS is a more suitable suspending medium for erythrocytes with respect to all of the loading parameters studied. The modified HBSS, having almost all of the vital factors for erythrocytes, provides better conditions for erythrocytes during the loading process and, probably, prevents to some extent the escape of intracellular elements from the erythrocytes at the point of lysis and for the entire process. This, in turn, ensures a longer in vivo life span for carrier erythrocytes, a hypothesis that must be proved by more studies.

Table 1

Concentrations of Enalaprilat Determined in Supernatant on Incubation of Intact Erythrocytes with Isotonic Solutions of Drug[a]

Initial Added Concentration	Supernatant Concentration		
	0 min	30 min	60 min
1 μg/ml	0.91 (0.11)[b]	1.01 (0.12)	0.91 (0.23)
8 μg/ml	7.48 (0.11)	7.61 (0.60)	7.71 (0.51)
80 μg/ml	79.47 (3.52)	77.55 (1.68)	78.26 (4.62)
800 μg/ml	772.98 (18.77)	769.79 (28.81)	781.43 (8.55)

[a] Incubation temperature 37°C.
[b] Mean (SD); $n = 3$.

Table 2

Effect of Type of Suspending Medium on the Loading Parameters of Enalaprilat in Human Erythrocytes

	K^+-Reversed HBSS	PBS
Loaded amount (μg)	241.90 (9.33)[a]	193.52 (8.02)
Efficiency of entrapment (%)	40.32 (1.56)	32.25 (3.00)
Cell recovery (%)	71.66 (3.44)	63.61 (5.31)

[a]HBSS = Hank's balanced salt solution; PBS = phosphate buffered saline. Mean (SD); $n = 3$.

Volume of Drug Solution

The effect of the volume of drug solution added during the encapsulation process on the loading parameters is shown in Fig. 1. An interesting consistency was found between these findings and the macroscopically detected point of lysis, at which the sudden increase in loaded amount and efficiency of entrapment occurs exactly when the transparency of erythrocyte suspension increases remarkably. Thus, the macroscopic evidence for the point of lysis can be used successfully for the detection of the lysis point. Furthermore, since both the loaded amount and the efficiency of entrapment were shown to be optimum with 700 μl of drug solution, which is enough to ensure the achievement of the lysis point. This volume was selected as the optimum volume to be added during the process.

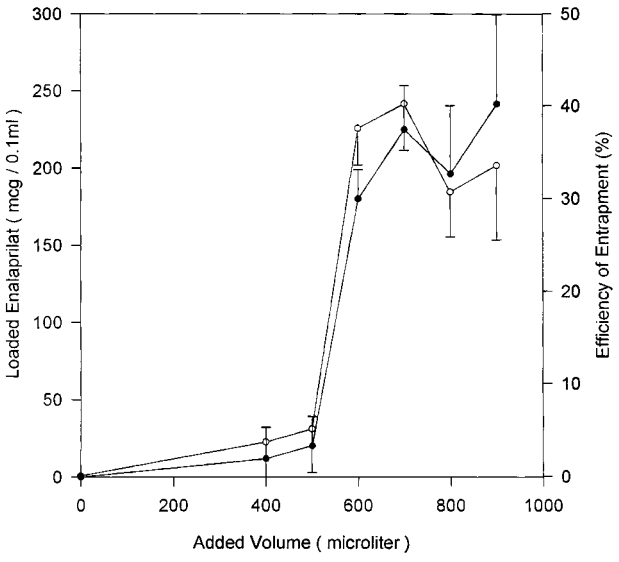

Figure 1. Effect of volume of added enalaprilat solution (8 mg/ml) on ● loaded amount and ○ efficiency of entrapment.

Method of Addition of Drug Solution

As it can be seen in Table 3, the best results were obtained when three successive portions of 230 μl each were used. However, for practical convenience, one can use three portions of 250 μl (considering the volume correction).

Concentration of Drug Solution

The effect of enalaprilat concentration on the loaded amount and efficiency of entrapment is shown in Fig. 2. According to this plot, it becomes clear that, although the loaded amount of drug is related directly to the concentration of drug solution throughout the range studied (it is noticeable that, as a result of limited water solubility of enalaprilat, concentrations higher than 8 mg/ml could not be achieved), the efficiency of entrapment increased up to a concentration of 4 mg/ml; beyond that, a declining trend was seen. However, while the use of concentrations higher than 4 mg/ml resulted in some lower efficiencies of entrapment, the concentration selected to use during the process was 8 mg/ml, mainly because of the higher absolute amount of loaded drug in unit volume of packed carrier cells, a parameter that is critical for dose adjustment during in vivo studies of this delivery system.

pH of Drug Solution

The effect of pH of enalaprilat aqueous solution on loading parameters is shown in Table 4. The acidic pH of the aqueous solution of enalaprilat (pH 2.8) might affect the structure and physiology of the erythrocyte membrane. So, it seems possible that the erythrocyte behavior during the loading procedure could be altered by the pH of the surrounding medium. When using a drug solution with a pH value adjusted to plasma pH (7.4), a total volume of 1 ml of drug solution brought the cells to the point

Table 3

Effect of Method of Addition of Enalaprilat Solution on the Loading Parameters of Drug in Human Erythocytes

	100 µl (7 times)	230 µl (3 times)[a]	350 µl (2 times)	700 µl (1 time)
Loaded amount (µg)	230.91 (9.43)[b]	246.87 (36.63)	219.65 (30.65)	188.27 (4.43)
Efficiency of entrapment (%)	41.23 (1.68)	44.72 (6.54)	39.31 (5.60)	33.62 (0.79)
Cell recovery (%)	67.54 (5.47)	68.88 (3.27)	60.78 (4.11)	52.10 (1.13)

[a] With volume correction.
[b] Mean (SD); $n = 3$.

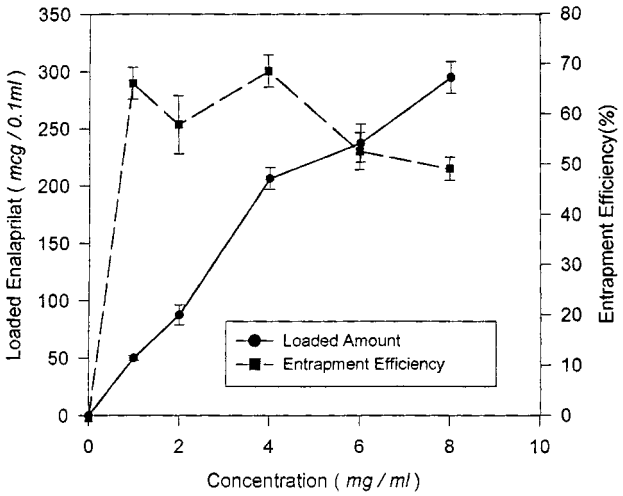

Figure 2. Effect of concentration of enalaprilat solution on ● loaded amount and ○ efficiency of entrapment.

of lysis (instead of 0.75 ml in the case of unchanged acidic solution). Therefore, it seems that the pH of drug solution has some effects on the erythrocyte biology. However, as can be seen in Table 3, in spite of the higher volume of drug solution that was added in the former case, the loaded amount of drug was not increased significantly. Furthermore, because of the higher total amount of drug added during the process, the efficiency of entrapment decreased in this case. The final cell recovery also was not affected by the pH of the drug solution.

Centrifugation Temperature

A comparison of loading parameters when all centrifuge steps were carried out at room temperature and 4°C is made in Table 5. The data shown in this table indicate that the temperature of the reaction mixture during several centrifuge steps has no profound effect on loading efficiency. In addition, using the ambient temperature, the erythrocytes may not subjected to any shock due to rapid changes in their temperature during the entire loading procedure, which has been pointed out by Pitt et al. (24).

Table 4

Effect of pH of Enalaprilat Solution on the Loading Parameters in Human Erythrocytes

	Unchanged Solution (pH = 2.8)	Neutralized Solution[a] (pH = 7.4)
Loaded amount (µg)	274.29 (8.08)[b]	272.39 (13.47)
Efficiency of entrapment (%)	45.57 (1.56)	34.05 (1.68)
Cell recovery (%)	65.48 (0.83)	63.93 (4.89)

[a] In this case, a total volume of 1 ml of drug solution was used during the loading process.
[b] Mean (SD); $n = 3$.

Table 5

Effect of Centrifuge Temperature During the Encapsulation of Enalaprilat in Human Erythrocytes on Loading Parameters

	Room Temperature	4°C
Loaded amount (μg)	243.54 (11.57)[a]	251.19 (9.05)
Efficiency of entrapment (%)	40.59 (1.93)	41.87 (1.51)
Cell recovery (%)	67.87 (3.74)	71.77 (5.22)

[a] Mean (SD); $n = 3$.

Packed Density of Cells

The loading parameters of four groups of erythrocytes with different packed densities are shown in Table 6. From the data, it becomes clear that the packed density of initial cells (in other words, the age of erythrocytes) used for encapsulation has no significant effect on the loading parameters. Interestingly, this was not the case when the preswelling step was omitted; in fact, our preliminary experiments showed that the nonswollen erythrocytes with different packed densities encapsulate, to some extent, different amounts of enalaprilat. Thus, it may be that preswelling has some unique effect in producing uniform cells to which approximately equal amounts of drug could enter at the point of lysis. Pitt et al. (24) also reported this type of modification on preswelling of erythrocytes.

Process Steps

The role of some of process steps (i.e., preswelling, lysate layer addition, and reannealing) in the entire loading procedure can be evaluated by considering the data shown in Table 7. These findings indicate that, first, although the omission of lysate layer addition and reannealing steps does not have any notable effect on the loading parameters, these steps can be excluded from the entire process only if their effects on the physiology of resealed erythrocytes (and, as a result, on the life span of carrier cells) can be studied. Second, the omission of the preswelling step, both alone and in combination with the lysate layer addition step, since it has no significant effect on the loaded amount, decreases the efficiency of entrapment due to the higher volume of enalaprilat solution needed to bring the cells to the point of lysis (1 ml vs. 0.75 ml used in the usual method) (Table 7). The lower value of cell recovery in these cases is another discouraging factor for these modifications. Third, preparation of the lysate layer using an aqueous solution of enalaprilat instead of water decreases the loaded amount due to the lower total volume of drug solution needed for achievement of the lysis point (0.5 ml vs. 0.75 ml used in the usual method). This takes place presumably because of the pH of the lysate layer, which is acidic when drug solution is used to prepare the lysate layer and causes the membrane to be more fragile. To prove this, as another experimental run, we prepared the lysate layer using neutralized drug solution. As expected, the cells again showed their usual nature. However, even in the last case, the loading parameters did not differ significantly from the usual method. This observation suggests that a limiting factor prevents the erythrocytes from taking more drug molecules at the point of lysis regardless of the availability of relatively more drug for cells. Generally,

Table 6

Effect of Density of Human Erythrocytes Used for Enalaprilat Encapsulation on the Loading Parameters[a]

	Group 1	Group 2	Group 3	Group 4
Loaded amount (μg)	241.15 (7.14)[b]	205.50 (11.10)	239.06 (5.54)	201.31 (7.23)
Efficiency of entrapment (%)	40.19 (1.19)	34.25 (1.85)	39.84 (0.92)	33.55 (1.21)
Cell recovery (%)	64.85 (5.12)	76.25 (3.27)	71.84 (2.58)	71.59 (7.08)

[a] The ranking order of groups is from the less-dense erythrocytes to the more dense ones.
[b] Mean (SD); $n = 3$.

Table 7

Effect of Changes of the Process Steps on Loading Parameters of Enalaprilat in Human Erythrocytes

	Loaded Amount (μg)	Efficiency of Entrapment (%)	Cell Recovery (%)
Usual method	249.76 (33.11)[a]	41.63 (5.52)	70.52 (7.28)
Without lysate layer	240.40 (17.20)	40.07 (2.87)	54.50 (4.02)
Without preswelling[b]	218.14 (9.03)	27.30 (1.13)	59.08 (4.21)
Without lysate layer and preswelling[b]	230.86 (18.16)	30.78 (2.42)	57.06 (2.50)
Without reannealing	222.60 (12.13)	37.1 (2.02)	65.02 (11.01)
Modified lysate layer[c]	184.48 (5.32)	38.43 (1.11)	65.72 (8.02)

[a] Mean (SD); $n = 3$.
[b] In these cases, a total volume of 1 ml of drug solution was used during the loading process.
[c] In this case, the lysate layer was prepared using enalaprilat aqueous solution (8 mg/ml).

these tests provide some useful evidence for confirmation of the necessity of all process steps for obtaining the best results from the loading process.

Mechanism of Entrapment

As shown in Table 8, for an experimental run, a total amount of about 4 mg of enalaprilat added during the encapsulation procedure was discarded as three washing solutions. At the same time, the total amount of drug remaining in the erythrocytes was 2.4 mg/ml or about 1.6 mg per total packed cells recovered after the loading process (cell recovery = 67.36%). The total volume of the reaction mixture was 2.25 ml at the point of resealing (this volume consisted of 1.2 ml for swollen cells, 0.2 ml for hemolysate, 0.75 ml for drug solution added in three steps, and 0.1 ml for hypertonic resealing solution), from which 0.6736 ml (about 30%) belonged to the carrier cells. Accordingly, it may be expected that, if the distribution of drug between the intracellular and extracellular fractions would be governed only by a simple concentration gradient–based diffusions from the total amount of 6 mg of added drug during the process, about 1.8 mg would be entrapped in the erythrocytes, and the rest (i.e., 4.2 mg) would be discarded as unentrapped drug.

In fact, as shown in Table 9, this is true, by a remarkable confidence (above 90%), and one can say that the partitioning of drug at the lysis point only depends on the volume fraction of cells in the suspension. Therefore, as it has been shown by the results of the incubation test of intact erythrocytes with enalaprilat, the erythrocyte membrane has no appreciable active role in the uptake of enalaprilat, so the drug only passes via the pores made in the membrane, on hemolysis, inward and outward the erythrocytes so that the concentration of drug inside and outside the cells becomes equal.

However, it should be noted that, according to the re-

Table 8

Entrapped and Unentrapped Amounts of Enalaprilat at the End of the Encapsulation Process

Fraction	Volume (ml)	Enalaprilat Concentration (μg/ml)	Total Enalaprilat Amount (μg)
First washing solution	11	307.10 (1.99)[a]	3378.1 (21.86)
Second washing solution	10	52.14 (1.65)	521.4 (16.55)
Third washing solution	10	12.34 (0.3)	123.4 (0.28)
Final packed cells	0.6736 (1.97)	2413.40 (52.9)	1625.67 (35.62)

[a] Mean (SD); $n = 3$.

Table 9

Recoveries of Enalaprilat as Entrapped, Unentrapped, and Total Drug After the Encapsulation Process in Human Erythrocytes

Fraction	Expected Amount (μg)	Measured Amount (μg)	Recovery[a]
Entrapped fraction	1796.27	1625.67 (35.62)[b]	90.48 (1.98)
Unentrapped fraction	4203.73	4022.90 (38.69)	95.70 (0.92)
Total	6000	5648.57 (44.37)	94.14 (0.74)

[a] The ratio of measured amount to expected amount expressed as percentage.
[b] Mean (SD); $n = 3$.

sults of another part of this study (see process steps section), the intracellular concentration of enalaprilat increases to a limited degree; above this, regardless of the increase of drug concentration in the extracellular compartment, the entrapped drug remains constant to a considerable extent. Baker (27) suggested that a transient permeable state of the erythrocyte membrane occurs after the onset of hemolysis. Then, on resealing, the pores are closed, and the drug is ''entrapped'' in the erythrocytes. The mechanism proposed here is in agreement with results published by others (13,18,20,27).

Table 10

*Loading Parameters of Encapsulation Method of Enalaprilat in Human Intact Erythrocytes (*n = *6)*

	Mean	SD
Loaded amount (μg)	242.66	9.38
Efficiency of entrapment (%)	40.44	1.56
Cell recovery (%)	70.44	2.35
Swelling index[a]	122.14	4.32

[a] The percentage ratio of hematocric value of erythrocytes after the swelling step to that of initial cells.

Loading Parameters

The average loading parameters of enalaprilat in intact human erythrocytes are shown in Table 10. The loaded amount of enalaprilat is conceivable in comparison to those values reported in the literature for a variety of drugs (2,21,22,24), which can ensure sufficient entry of drug to the body on reinjection of a fairly low volume of packed cells. A cell recovery of about 70% is comparable to results published by others (14,23,25).

Process Validation Tests

Table 11 shows that the encapsulation process for enalaprilat has a considerable degree of intrarun and interrun reproducibility. Also, as shown in Table 12, the intersubject variation of the loading procedure is fairly low even when samples taken from subjects of both sexes were included in study.

The recovery of the encapsulation process is shown in Table 9 with respect to entrapped, unentrapped, and total drug. These data provide a reasonable basis for investigation of the mechanism of entrapment and indicate a remarkable degree of accuracy for the whole process.

Table 11

*Between-Run and Within-Run Variations of Loading Parameters of Enalaprilat in Human Intact Erythrocytes (*n = *6)*

	Between Run			Within Run		
	Mean	SD	CV%	Mean	SD	CV%
Loaded amount (μg)	244.84	12.11	4.95	238.18	8.68	3.64
Efficiency of entrapment (%)	40.81	2.01	4.93	39.70	1.45	3.65
Cell recovery (%)	70.44	2.35	3.34	69.85	4.21	6.03

Table 12

Loading Parametes of Enalaprilat in Erythrocytes of Two Male (No. 1 and No. 2) and Two Female (No. 3 and No. 4) Volunteers[a]

	Subject 1	Subject 2	Subject 3	Subject 4
Loaded amount (μg)	280.90 (22.0)[b]	240.65 (22.08)	241.70 (5.80)	183.27 (23.84)
Efficiency of entrapment (%)	46.82 (3.67)	40.11 (3.69)	40.28 (0.97)	30.54 (3.97)
Cell recovery (%)	70.59 (7.28)	66.44 (3.13)	67.36 (1.97)	68.81 (2.40)

[a] Aged 27–36 years.
[b] Mean (SD); $n = 3$.

Also, the total recovery of enalaprilat after the loading process (about 27%) is a promising result considering the methodological limitations and the clinical potentials of this delivery system.

CONCLUSION

The methodological variables of the hypotonic preswelling method for the loading of human intact erythrocytes by drugs and other bioactive agents were evaluated with respect to their corresponding effects on the loading parameters (i.e., loaded amount, efficiency of entrapment, and cell recovery) using enalaprilat as a model drug. Knowledge of the optimal conditions required by a process to lead to the best results allows design of a suitable encapsulation procedure for a particular drug with some minor modifications, if necessary, and this study can serve as a model for such evaluations. In addition, the mechanism of entrapment of enalaprilat in intact human erythrocytes was exploited, and it was shown that the probable mechanism is the entry of drug to the cells via the pores made on the cell membrane at the point of hemolysis. Then, on closure of pores by hypotonic resealing, the drug is trapped in the cells. Development of this intravenous delivery system for enalaprilat may not only be remarkable therapeutically, but also may provide some valuable information about the possibility of modified-release delivery of peptide drugs using this system owing to the structural similarities of this drug with peptides. Finally, validation tests were carried out on the loading method, and the results indicate a remarkable degree of accuracy, precision, and reproducibility for the method. Further studies have been performed to characterize the carrier erythrocytes; the results will be published separately.

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REFERENCES

1. J. G. Garrison and M. J. Peach, Renin and angiotensin, in *The Pharmacological Basis of Therapeutics* (A. Goodman Gillman, T. W. Rall, A. S. Nies, and P. Taylor, Eds.), Pergamon, New York, 1991, pp. 749–763.
2. C. Ropars, M. Chassaigne, and C. Nicolau (Eds.), *Advances in Biosciences*, Vol. 67, Pergamon, Oxford, UK, 1987.
3. R. Green, *Methods in Enzymology*, Vol. 149, Academic, San Diego, CA, 1987.
4. U. Zimmermann, Cellular drug-carrier systems and their possible targeting, in *Targeted Drugs* (E. P. Goldberg, Ed.), John Wiley and Sons, New York, 1983, pp. 153–200.
5. D. A. Lewis, Red blood cells for drug delivery, Pharm. J., 233, 384–385 (1984).
6. D. A. Lewis and H. O. Alpar, Therapeutic possibilities of drugs encapsulated in erythrocytes, Int. J. Pharm., 22, 137–146 (1984).
7. H. G. Eichler, S. Gasic, B. Daum, S. Bacher, and G. Steger, In vitro drug release from human carrier erythrocytes, in *Advances in Biosciences*, Vol. 67 (C. Ropars, M. Chassaigne, and C. Nicolau, Eds.), Pergamon, Oxford, UK, 1987, pp. 11–15.
8. K. Adriaenssens, D. Karcher, A. Lowenthal, and H. G. Terheggen, Use of enzyme-loaded erythrocytes in in vitro correction of arginase-deficient erythrocytes in familial hyperargininemia, Clin. Chem., 22(3), 323–326 (1976).
9. H. O. Alpar and D. A. Lewis, Therapeutic efficacy of

asparaginase encapsulated in intact erythrocytes, Biochem. Pharmacol., 34(2), 257–261 (1985).
10. N. Talwar and N. K. Jain, Erythrocytes as carriers of metronidazole: in-vitro characterization, Drug Dev. Ind. Pharm., 18(16), 1799–1812 (1992).
11. W. N. Field, M. D. Gamble, and D. A. Lewis, Comparison of the treatment of thyroidectomized rats with free thyroxine and thyroxine encapsulated in erythrocytes, Int. J. Pharm., 51, 175–178 (1989).
12. E. Pitt and D. A. Lewis, The use of corticosteroids encapsulated in erythrocytes in the treatment of adjuvant induced arthritis in the rat, Biochem. Pharmacol., 32(22), 3355–3358 (1983).
13. G. M. Ihler, R. H. Glew, and F. W. Shnure, Enzyme loading of erythrocytes, Proc. Natl. Acad. Sci., 70(9), 2663–2666 (1973).
14. N. Talwar and N. K. Jain, Erythrocytes as carriers of primaquine-preparation: characterization and evaluation, J. Controlled Release, 20, 133–142 (1992).
15. E. Zocchi, U. Bennatti, L. Guida, M. Tonetti, G. L. Damonte, and A. D. Flora, Encapsulation of glucose oxidase in mouse erythrocytes: an experimental model of oxidant-induced cytotoxicity and a means for splenic targeting of carrier erythrocytes, in *Advances in Biosciences*, Vol. 67 (C. Ropars, M. Chassaigne, and C. Nicolau, Eds.), Pergamon, Oxford, UK, 1987, pp. 95–101.
16. U. Bennatti, E. Zocchi, M. Tonetti, L. Guida, and A. D. Flora, Comparative tissue distribution and metabolism of free versus erythrocyte encapsulated adriamycin in the mouse, in Advances in Biosciences, Vol. 67 (C. Ropars, M. Chassaigne, and C. Nicolau, Eds.), Pergamon, Oxford, UK, 1987, pp. 129–136.
17. U. Zimmermann, F. Riedman, and G. Pilwat, Enzyme loading of electrically homogeneous human red blood cell ghosts prepared by dielectric breakdown, Biochim. Biophys. Acta, 436, 460–474 (1976).
18. K. Kinosita and T. Y. Tsong, Formation and resealing of pores of controlled size in human erythrocyte membrane, Nature, 268, 438–440 (1977).
19. K. Kinosita and T. Y. Tsong, Hemolysis of human erythrocytes by a transient electric field, Proc. Natl. Acad. Sci., 74(5), 1923–1927 (1977).
20. J. Deloach and G. Ihler, A dialysis procedure for loading erythrocytes with enzymes and lipids, Biochim. Biophys. Acta, 496, 136–145 (1977).
21. J. Deloach, R. L. Harris, and G. Ihler, An erythrocyte encapsulator dialyser used in preparing large quantities of erythrocyte ghosts and encapsulation of pesticide in erythrocyte ghosts, Anal. Biochem., 102, 220–227 (1980).
22. R. Kravtzoff, C. Ropars, M. Laguerre, J. P. Muh, and M. Chassaigne, Erythrocytes as carriers for L-asparaginase. Methodological and mouse in-vivo studies, J. Pharm. Pharmacol., 42, 473–476 (1990).
23. A. Zanella, F. Rossi, L. Sabbioneda, V. Russo, A. Brovelli, F. De Cal, S. Fargion, G. Fiorelli, and G. Sirchia, Desferrioxamine loading of red cells for transfusion, in *Advances in Biosciences*, Vol. 67 (C. Ropars, M. Chassaigne, and C. Nicolau, Eds.), Pergamon, Oxford, UK, 1987, pp. 17–27.
24. E. Pitt, C. M. Johnson, D. A. Lewis, D. A. Jenner, and R. E. Offord, Encapsulation of drugs in intact erythrocytes: an intravenous delivery system, Biochem. Pharmacol., 32(22), 3359–3368 (1983).
25. H. O. Alpar and W. J. Irwin, Some unique applications of erythrocytes as carrier systems, in *Advances in Biosciences*, Vol. 67 (C. Ropars, M. Chassaigne, and C. Nicolau, Eds.), Pergamon, Oxford, UK, 1987, pp. 1–9.
26. H. Tajerzadeh and M. Hamidi, A simple HPLC method for quantitation of enalaprilat, Pharm. Biomed. Anal. (2000).
27. R. F. Baker, Entry of ferritin into human red cells during hypotonic haemolysis, Nature, 215, 424–425 (1967).