Drug Development and Industrial Pharmacy, 23(10), 999-1006 (1997)

RESEARCH PAPER

Magnetically Guided Rat Erythrocytes Bearing Isoniazid: Preparation, Characterization, and Evaluation

Sanjay Jain, Sanjay K. Jain, and V. K. Dixit

Pharmaceutics Research Laboratory, Department of Pharmaceutical Sciences, Doctor Harisingh Gour Vishwavidyalaya, Sagar (M.P.) 470003 India

ABSTRACT

Rat erythrocytes were loaded with isoniazid and magnetite by the preswell technique. Various parameters such as drug concentration, magnetite concentration, and volume of aqueous solution were optimized to study the maximum loading of drug into erythrocytes (67.2 ± 1.6%). The loaded cells were characterized for drug and magnetite content, hemoglobin content, percent cell recovery, morphology, osmotic fragility, turbulence shock, in-vivo drug and hemoglobin efflux, and magnetic responsiveness. No appreciable detrimental effect on cell morphology, osmotic fragility, and turbulence shock in comparison to normal cells was noted. However, drug and magnetite showed little detrimental effect on cells. Drug release from these systems followed approximately zero-order kinetics. The drug- and magnetite-loaded cells effectively responded to an external magnetic field of 8.0 ± 1.0 K.Oe. The in-vivo studies showed that an erythrocyte-based delivery system has potential to increase drug concentration many fold at the target site under influence of an external magnetic field. The drug-loaded erythrocytes appeared to be promising carriers of isoniazid to infected organ/tissue.

INTRODUCTION

Recently, considerable research has been directed toward the development of drug carriers for better therapeutic performance. Erythrocytes offer many advantages over other cellular carriers in selective and effective delivery of the bioactive agents for human and veterinary applications. Erythrocytes are nonimmunogenic and biodegradable; they freely circulate throughout the body and offer ease of preparation; they have the capacity to carry large amounts of drug; and can behave as a slow-release, long-acting system (1,2). An erythrocyte-based delivery system has the potential to improve the quality of drug therapy compared to

conventional methods because it minimizes frequent dosing while maintaining a constant therapeutic level at the infected area.

The use of erythrocytes as a potential delivery system for drugs, enzymes, and other compounds has been reported (3), and a number of methods have been studied to incorporate drug into the cells (4). The therapeutic applications include targeting of the enclosed drug to the desired site of action such as a tumor or diseased tissue or organ (5). Gluteraldehyde and similar treatment allows the erythrocytes to target or localize in liver or spleen (6,7). Sprandel et al. (8) have recently reported loading of magnetite and a drug into erythrocytes, which can then be used for the targeting of the drug to the infected area with the help of an external magnetic field.

Isoniazid is a first-line agent in the treatment of tuberculosis, with half-life of 1.0–1.5 hr in fast acetylators. It is administered two or three times a day and used in long-term therapy. It has a tendency to develop hepatotoxicity and neurotoxicity when administered orally (9). It is difficult to obtain a constant therapeutic blood level in this class of population. Therefore, the development of magnetically guided erythrocytes bearing isoniazid for controlled and selective delivery of drug to targeted site would be beneficial.

MATERIALS AND METHODS

Materials

Isoniazid was supplied by Pfizer (I) Ltd., Bombay, India. Ferrous sulfate, sodium hydroxide, acetonitrile, sodium chloride, and all other ingredients were of analytical grade, supplied by Loba Chemie Pvt. Ltd., Bombay, India.

Separation of Erythrocytes

Blood was collected by cardiac puncture of animals (male albino rats weighing 175–200 g). The whole blood was centrifuged at 2250 rpm for 5 min at 4°C in a refrigerated centrifuge (TC-4100, Sico, India). The serum and buffy coat were removed carefully and erythrocytes were washed three times with saline phosphate buffer (SPB) pH 7.4 before being stored at 4°C in SPB.

Magnetite Preparation and Coating

Magnetite (Fe_3O_4) was prepared following the method reported by Malaiya and Vyas (10) and was coated with 0.1% silicon oil in ether to reduce its hemolytic and cytotoxic effect on cell's wall (11).

Loading of Erythrocytes

The drug and magnetite were loaded in erythrocytes using the modified preswell method reported by Pitt and Hattori (12). Packed erythrocytes (1 ml) were mixed with 4 ml of SPB (pH 7.4). The tonicity was adjusted to 0.65% of normal value. The suspension was centrifuged at 600 rpm for 5 min. The swollen cells were collected and the supernatant was discarded. The cells' hemolysate (200 µl, prepared by lysing packed erythrocytes using water as 1:1 ratio) was layered over the top of the swollen cell. Without disturbing the hemolysate layer, a 200-µl aqueous injection of isoniazid and 100 µl (2% w/w) of presonicated silicon coated magnetite nanoparticles (11) were layered on the top of the mixture, and the tube was gently mixed by occasional inversion. The hemolysate was layered so as to provide an osmotic barrier against the hypotonic drug and magnetite solution. The addition of the stated amount of drug solution and magnetite suspension was sufficient to bring the swollen cells to the state of lysis. After the cells were loaded, hypertonic SPB solution was added to restore the isotonicity. The loaded erythrocytes were subsequently incubated at 37 ± 1°C for 30 min to allow annealing of the cells. The drug-loaded cells were recovered by centrifugation and washed three times with SPB to remove any surface-adhering drug. The entrapped particles were removed from the cell suspension with the help of horseshoe magnet (1.5 ± 0.2 K.Oe.).

Effect of Process Variables

The effects of drug concentration (200 µl of 2, 3, 4, 5, and 6 mg/ml), volume of drug solution (100, 200, and 300 µl of 4 mg/ml solution), and magnetite concentration (200 µl of 1, 2, 3, and 5% w/w presonicated at 50 Kc/sec for 60 min) on percent drug loading, hemoglobin content, and cell recovery were studied. These parameters were optimized for effective loading of drug and magnetite into erythrocytes.

Drug and Magnetite Content

Packed, loaded erythrocytes (0.2 ml) were deproteinized with acetonitrile (2 ml) after centrifugation at 2800 rpm for 10 min. A horseshoe magnet (1.5 ± 0.2 K.Oe.) was placed adjacent to the base of the centrifuged tube to retain magnetite. The clear supernatant was withdrawn and filtered using a 0.45-µm filter

(Kumar and Co., Bombay, India). The amount of drug was estimated spectrophotometrically at 280 nm in the supernatant (13). The magnetite content was determined using the method reported by Gallo et al. (14).

Hemoglobin Content

The drug-loaded cells in suspension (5% hematocrit in SPB) were centrifuged at 1800 rpm for 2 min (after being stored at 4°C) and absorbance was determined at 540 nm (a) of separated clear supernatant. The supernatant of washed RBC was taken as background solution (b). The absorbance of lysed RBC (100% hemoglobin released cells) was taken at 540 nm (c). The percent hemoglobin was calculated using the formula (15)

$$\text{percent hemoglobin} = [(a - b)/c] \times 100$$

Percent Recovery and Morphology

Percent cell recovery was determined using a hemocytometer (Neubar, Japan) by counting number of cells per cubic millimeter of packed erythrocytes before and after loading of drug and magnetite. Isoniazid-loaded cells (ILCs) and isoniazid loaded magnetic cells (ILMCs) were studied for morphological evaluation using a phase contrast microscope (Wild Leitz, Biomed, Germany).

Osmotic Fragility

Osmotic fragility study was performed for normal cells, ILCs, and ILMCs following the method described by Sprandel and Zollner (16). Erythrocytes (0.5 ml of packed cells) were incubated in sodium chloride solutions at different strengths (0.9–0.1%) for 10 min at 37°C. The suspension was centrifuged and the supernatant was estimated spectrophotometrically for percent hemoglobin leakage.

Turbulence Shock

The cells were passed through a 22-gauge hypodermic needle at the rate of 8–10 ml/min and hemoglobin efflux was estimated after a fixed number of passes (17).

In Vitro Drug and Hemoglobin Efflux

Loaded cells were incubated at 37 ± 1°C in SPB pH 7.4 (50% hematocrit) in a metabolic bath with occasional shaking, and samples were removed at different time intervals with the help of a hypodermic syringe fitted with a 0.45-μm membrane filter. Drug and hemoglobin content were then estimated (18).

Magnetic Responsiveness

Magnetic responsiveness was determined by counting cells per milliliter of suspension at a trap point under a magnetic field of 8.0 ± 1.0 K.Oe. Those not responding to the magnetic field were passed unentrapped and collected in a beaker (19).

In Vivo Evaluation

Isoniazid Tissue Distribution Studies

The male albino rats (weighing 175–200 g) were used to study the tissue distribution of isoniazid. Animals were divided into four groups of three rats each. One milliliter of isoniazid solution (equivalent to 225 μg) was administered intravenously to group 1 animals through a femoral vein, while group 2 and group 3 animals received ILCs and ILMCs (equivalent to 225 μg INH), respectively. Group 4 was used as a control. After the administration of drug and its formulation, the animals were sacrificed at 1, 4, and 12 hr and liver, lung, spleen, and kidney were isolated. Simultaneously, blood was collected from heart. The isolated organs were rinsed with cold normal saline, blotted, and weighed. The organs were then macerated in ethanol and centrifuged. The drug content was determined in the supernatant after filtration.

In Vivo Magnetic Responsiveness

Adult male albino rats weighing 175–200 g were anesthetized using anesthetic ether. During cannulation the anesthesia was maintained using halothane (19). The anesthetized rats were placed dorsally and the caudal artery was exposed and catheterized. The tail was marked into four segments. Segment A was the injection site; segment B, the pretarget site; segment C, the preselected target site; and segment D, the post-target site. Isoniazid (equivalent to 225 μg), contained in magnetite-bearing erythrocytes, was injected through the injection site at speed of 0.5–0.7 cm/sec, comparable to blood flow rate. Before dose administration, a bipolar magnet of field strength 8.0 ± 1.0 K.Oe. was placed at the preselected target site (segment C). The magnet was left for 15, 30, and 60 min. The animal was then immediately sacrificed. Three rats were used each time. Samples of blood were collected from the heart and allowed to clot. The liver, lung, spleen, and kidney were removed, rinsed with cooled normal saline, blotted, and homogenized in ethanol (2 ml/g tissue) using

a laboratory homogenizer (Remi, India). The tail was cut into four equal parts, and each tail piece was cut into small pieces, then crushed and stored overnight at 4°C. The clear supernatant liquid obtained after centrifugation and filtration was subjected to drug content determination (13). The drug localization index was determined at various time intervals using the equation described by Gupta and Hung (20):

$$\text{drug localization index} = \frac{\begin{array}{c}\%\text{ drug concentration in tissue}\\ \text{at time } t \text{ after administration}\\ \text{of drug and magnetite-loaded cells}\end{array}}{\begin{array}{c}\%\text{ drug concentration in tissue}\\ \text{at time } t \text{ after administration}\\ \text{of drug solution}\end{array}}$$

RESULTS AND DISCUSSION

The preswell loading technique reported by Pitt and Hattori (12) was successfully used to load isoniazid and magnetite into erythrocytes. The preswell technique consists of first swelling the cells without lysis by placing them in slightly hypotonic media. The gentle swelling of the cells was followed to the point of lysis by adding appropriate amounts of aqueous drug and magnetite. A little modification with respect to use of SPB for preswelling the cells in place of Hank's balanced salt solution (HBSS) was made. Entrapment of drug/magnetite took place when tonicity was maintained and cells were incubated for a desired period of time. The gentle swelling of cells resulted in good retention of cytoplasmic constituents and good in vivo survival times (21).

The various process parameters that could affect the loading of drug and magnetite were optimized. Maximum loading was recorded at 4 mg/ml of drug concentration. At this concentration, cells were observed to be discoid in shape. As the concentration increased above 4 mg/ml, the percent loading, cell recovery, and hemoglobin content were observed to decrease (Table 1). At higher concentrations, crenated cells were obtained with a few lumps of fragmented membrane or bunches of ruptured cells. This may be due to hemolytic effect of drug and magnetite on the cell wall.

The 200 μl solution was required to bring the swollen cells to the point of lysis, which could be tested by observing a thin layer of white ghost on top of packed cells. Upon addition of 100 μl aqueous solution of INH, only 25.1% of drug could be encapsulated in cells, whereas on addition of 200 μl solution of INH, 67.2% drug entrapment was recorded with 73.2% cell recovery and 80.3% hemoglobin content. On further addition of drug solution (300 μl), an almost complete lysis of cells was observed, which could be due to the detrimental affect of excessive aqueous drug solution of cells (Table 1). This was seen as an appearance of ruptured and aggregated cells under a light microscope.

Table 1

Encapsulation Parameters of Drug- and Magnetite-Loaded Cells

Parameter	% Loading	% Cellular Recovery	% Hemoglobin Content	% w/w Magnetite
Drug conc. (mg/ml)				
2	60.3 ± 0.8	79.2 ± 1.8	81.2 ± 1.2	—
3	63.2 ± 1.2	71.4 ± 2.2	75.1 ± 1.4	—
4	67.2 ± 1.6	64.2 ± 2.4	69.2 ± 1.9	—
5	62.0 ± 2.1	46.4 ± 1.6	61.2 ± 1.2	—
6	59.0 ± 0.9	32.4 ± 1.2	46.2 ± 1.7	—
Vol. of aqueous drug solution (μl)				
100	25.1 ± 1.6	86.0 ± 2.3	92.2 ± 2.1	—
200	67.2 ± 1.2	73.2 ± 1.2	80.3 ± 2.2	—
300	2.4 ± 0.7	4.0 ± 0.4	6.0 ± 1.1	—
Magnetite conc. % w/w				
1	66.8 ± 0.7	80.2 ± 1.4	83.2 ± 1.6	8.2 ± 1.5
2	67.1 ± 1.6	73.2 ± 1.6	78.2 ± 2.4	12.8 ± 2.6
3	44.2 ± 2.2	60.2 ± 2.4	76.3 ± 2.6	6.9 ± 2.1
5	8.0 ± 2.4	14.7 ± 1.6	20.4 ± 3.1	2.3 ± 2.2

Values represented as mean ± *SE* (n = 3).

The effect of magnetite concentration on its pay load, drug entrapment, cellular recovery, and cellular hemoglobin content showed that best results were obtained with 2% w/w of magnetite. Above 2% magnetite concentration, the percent drug entrapment, cell recovery, and cellular hemoglobin content were noted to decrease significantly (Table 1). This may be due to varying degrees of cytotoxicity and hemolytic activity of magnetite, which caused permanent deformity in the cellular structure of erythrocytes. It was found that when drug was loaded using optimized conditions, the majority of carrier cells appeared spherical as well as discoid in shape, while in the case of drug- and magnetite-bearing cells, few cells appeared of stomatocytic shape. These findings are similar with the result recorded by Sprandel et al. (8).

Isoniazid- and magnetite-loaded erythrocytes were formulated using optimized conditions and characterized for morphology, osmotic fragility, turbulence shock, in vitro drug and hemoglobin release, and magnetic responsiveness.

The discoid appearance of cells of all the preparations indicated that the drug caused no cell deformations when the drug was loaded under optimized drug concentration, whereas on loading drug along with magnetite, few stomatocytic erythrocytes with some membrane perturbation were seen.

The osmotic fragility study of normal erythrocytes showed 50% hemolysis at 0.35% sodium chloride, while ILCs and ILMCs exhibited 50% hemolysis at 0.43% and 0.53%, respectively. Osmotic fragility was less resistant to osmotic stress, which may be due to the drug-borne osmotic pressure of cells. The drug- and magnetite-loaded erythrocytes demonstrated less resistance to osmotic fragility as compared with normal and drug-loaded erythrocytes. Slightly higher sensitivity of ILMCs toward osmotic stress may be due to the detrimental effect of magnetite on cell walls (Table 2).

The turbulence shock study showed that 50% of the hemoglobin was released from normal erythrocytes after 19 passes, while the same amount of hemoglobin release was noted after 18 and 12 passes in the cases of ILCs and ILMCs, respectively. ILMCs were found to be most sensitive to turbulence shock. This may be due to harmful effect of magnetite on cell walls (Table 2).

The in vitro drug and hemoglobin efflux profiles of ILCs and ILMCs were found to follow approximately zero-order kinetics. Fifty percent of INH was released in 4.7 hr from ILCs, while ILMCs released 50% INH in 3.5 hr, indicating more porous cellular wall of ILMCs. This could be due to detrimental effect of magnetite on the cell walls (Table 3).

The ILMCs, which are responsive to magnetic field, were isolated by localizing them at a trap point with a magnetic field of 8.0 ± 1.0 K.Oe. Fifty percent of the ILC population was found to be responsive to an external magnetic field after 4.2 hr (Table 4).

Table 2

Osmotic Fragility and Turbulence Shock Study of Loaded Cells

Parameter	% Hemolysis of Cells		
	Normal	ILCs	ILMCs
% Sodium chloride			
0.9	1.1 ± 0.6	4.6 ± 1.4	10.3 ± 0.5
0.8	1.7 ± 0.7	8.0 ± 2.4	19.1 ± 0.9
0.7	2.0 ± 1.1	10.3 ± 1.6	24.3 ± 0.4
0.6	2.6 ± 1.2	18.5 ± 1.3	37.2 ± 1.2
0.5	5.7 ± 1.4	40.5 ± 0.7	55.2 ± 1.2
0.4	7.0 ± 1.6	66.7 ± 1.1	84.2 ± 1.6
0.3	94.0 ± 1.8	96.0 ± 0.4	97.0 ± 2.1
0.2	96.0 ± 1.2	99.0 ± 1.2	99.1 ± 3.2
0.1	99.2 ± 0.6	99.6 ± 0.6	99.7 ± 0.6
No. of passes			
10	23.3 ± 1.2	35.4 ± 1.4	46.5 ± 0.9
20	56.2 ± 2.2	64.5 ± 1.2	80.2 ± 0.9
30	76.3 ± 2.6	88.2 ± 1.0	99.2 ± 0.2
40	93.2 ± 3.2	99.0 ± 0.7	—

Values represented as mean ± *SE* (n = 3).

Table 3

In Vitro Release of INH and Hemoglobin from ILCs and ILMCs

Time (hr)	% Drug/Hemoglobin Released From Cells			
	INH Loaded		INH and Magnetite Loaded	
	INH	Hemoglobin	INH	Hemoglobin
1	16.2 ± 1.2	5.2 ± 1.2	24.1 ± 1.2	9.2 ± 1.2
2	30.1 ± 1.1	12.1 ± 2.7	38.2 ± 1.1	16.4 ± 1.6
3	35.2 ± 1.6	17.3 ± 3.2	46.2 ± 0.7	22.4 ± 1.4
4	43.1 ± 1.7	21.4 ± 2.5	53.4 ± 0.9	27.2 ± 1.2
5	52.1 ± 1.2	24.3 ± 2.4	61.2 ± 0.5	32.2 ± 1.4
6	59.2 ± 0.7	28.2 ± 2.2	69.3 ± 0.6	35.2 ± 1.2
7	70.3 ± 0.2	31.2 ± 2.0	77.4 ± 1.2	38.2 ± 0.7
8	75.4 ± 0.1	33.2 ± 2.1	86.2 ± 1.1	40.2 ± 0.6
9	80.2 ± 2.7	36.5 ± 0.9	89.2 ± 1.6	46.2 ± 0.7

Values represented as mean ± *SE* (n = 3).

Tissue distribution of INH following intravenous administration of the formulation showed that after 1 hr of INH administration, maximum concentrations of 25.8% and 25.4% were recorded in liver from ILMCs (A) and ILCs (B), respectively. The concentration in spleen was found to be 10.9% and 10.7% for ILMCs and ILCs, respectively. Relatively smaller amounts of INH (i.e., 14% and 7.6%) were estimated from liver and spleen when the drug was administered in solution form (C). This shows that concentrations reached to higher levels in organs where phagocytosis is predominant. INH levels of 11.1% and 9.9% were found in lung after 1 hr following the administration of ILMCs and ILCs, respectively, while 4.9% INH was estimated when administered in solution. The amounts of INH in kidney were 5.9%, 5.7%, and 5% in preparations of ILMCs, ILCs, and drug solution, respectively. In the blood, the INH concentrations were 11.9%, 10.9%, and 20.19% for ILMCs, ILCs, and solution, respectively. These results showed the slow drug release from erythrocyte carriers (Table 5).

The INH concentrations in lung, kidney, and blood were found to decrease progressively, and after 12 hr less than 2.9% drug was estimated in lung, kidney, and blood invariably after the administration of any dosage form. Drug concentration in spleen was also reduced remarkably. Negligible concentration (less than 1.9%) was recorded in blood following drug solution administration as compared to the drug blood level (i.e. 1.9% and 2.4%) estimated after the administration of ILMCs and ILCs, respectively. This pattern could be described to the control and slow release of drug from both of the erythrocyte-based preparations.

The in vivo target specificity and selectivity of ILCs were evaluated by assessing INH content at the target site and other cutaneous and subcutaneous parts. Fifty-one percent of INH could be localized in the tail segment (C) within 1 hr following the application of magnetic field of 8.0 ± 1.0 K.Oe. The INH content in the rest of the tail segment was nearly nil. This indicates higher responsiveness and selectivity of preparation to the site of magnetic application. It is clear from the results that as the duration of magnetic field application increases, the INH concentration in liver decreases progressively and tends to accumulate at the site of magnet application (segment C). Control rats (without magnetic field application) showed 31.9%, 10.4%, 11.0%,

Table 4

Magnetic Susceptibility of INH-Loaded Magnetic Erythrocytes

Time (hr)	% Magnetic Response Remaining
1	92.2 ± 1.6
2	80.2 ± 2.4
3	67.2 ± 1.2
4	54.1 ± 1.6
5	40.4 ± 1.6
6	29.6 ± 1.2
7	22.9 ± 0.7
8	18.7 ± 0.9
9	6.9 ± 2.0

Values represented as mean ± *SE* (n = 3).

Table 5

Tissue Distribution of Isoniazid Following the Administration of ILMCs (A), ILCs (B), and INH Solutions (C)

Time (hr)	% INH Recovered														
	Liver			Lung			Spleen			Kidney			Blood		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
1	25.8	25.4	14.0	11.1	9.9	4.9	10.9	10.7	7.6	5.9	5.7	5.0	11.9	10.9	20.9
4	20.9	19.1	9.7	8.4	8.1	3.6	8.9	8.7	5.9	4.2	3.9	3.6	8.1	6.9	9.9
12	4.8	4.9	2.0	1.7	1.9	—	2.4	2.9	1.9	1.9	1.9	1.9	1.9	2.4	0.9

Table 6

In Vivo Targeting of INH and Magnetite-Loaded Erythrocytes

Duration of Magnetic Field (min)	% INH							
	Organ				Tail Segment			
	Liver	Lung	Spleen	Kidney	A	B	C	D
0	31.9	10.4	11.9	3.4	1.9	ND	1.95	ND
15	21.1	7.4	7.3	2.5	1.9	1.9	21.00	1.9
30	10.1	5.6	6.1	2.0	1.8	3.2	31.00	21.9
60	6.6	3.3	2.9	1.9	1.9	4.2	51.00	4.8

and 3.4% INH concentration in liver, lung, kidney, and spleen, respectively, while in the tail segment (A and C) less than 2% drug was estimated. The higher-level concentration is attributed to the fact that magnetic erythrocytes are removed and accumulated in liver and spleen by reticuloendothelial system (20). In case of reduced exposure to nontargetable tissues in presence of magnetic field for 60 min, only 6.6% drug was estimated in liver (Table 6).

The maximum drug targeting index was found to be 44.2 when the magnetic field was applied for 60 min and the minimum (16.4) was reached with 15 min of magnetic field application. The results demonstrated the enhancement of drug target index with increasing duration of magnetic field application of 8.0 ± 1.0 K.Oe. strength (Table 7).

Table 7

INH Localization Index

Duration of Magnetic Field (min)	Drug Targeting Index
15	16.4
30	32.3
60	44.2

CONCLUSION

We conclude that isoniazid can successfully be targeted at the infected area using drug-loaded magnetic cells under the guidance of an external magnetic field. However, further studies are required in order to optimize the dose of drug-loaded magnetic cells with different strengths and application times of the magnetic field.

ACKNOWLEDGMENTS

The authors are thankful to Pfizer (I) Ltd., Bombay, India for supply of isoniazid as a gift sample; Head of Department of Pharmaceutical Sciences, Dr. H. S. Gour Vishwavidyalaya, Sagar, M. P. India, for providing necessary facilities to carry out the present work; and C.S.I.R., New Delhi, India, for providing financial assistance for one of the authors.

REFERENCES

1. U. Zimmerman, G. Pilwat, and J. Holzo., J. Membrane Biol., 30, 135 (1976).
2. G. M. Ihler, R. H. Glew, and F. W. Schnure., Pro. Natl. Acad. Sci., 70, 2663 (1970).
3. J. R. DeLoach, Med. Rev., 6, 4876 (1986).

4. R. L. Juliano, ed., *Drug Delivery System*, Oxford University Press, London, 1980, p. 237.
5. N. Talwar and N. K. Jain, J. Contr. Rel., 20, 133 (1992).
6. N. Talwar and N. K. Jain, J. Microencapsulation, 9, 357 (1992).
7. M. Pinilla, J. A. Jordan, J. C. Diez, and J. Luque, Advances in the Biosciences, 92, 7 (1994).
8. U. Sprandel, Advances in the Biosciences, 92, 191 (1994).
9. F. S. K. Barar, ed., *Essential of Pharmacotherapeutics*, 2nd ed., S. Chand and Company, New Delhi, 1990, p. 702.
10. S. P. Vyas and A. Maliaya, J. Microencapsulation, 6, 493 (1980).
11. U. Zimmerman, Chem. Eng. News, 24, 21 (1975).
12. T. Pitt and K. Hattori, IRCS Med. Sci., 8, 89 (1980).
13. *British Pharmacopoeia*, Vol I, 1st ed., Her Majesty's Office, U.K., 1988, p. 317.
14. G. M. Gallo, P. K. Gupta, C. T. Hung, and D. G. Perrier, J. Pharm. Sci., 78, 190 (1989).
15. D. H. Mitchell, T. James, and C. A. Kruse, Biotechnol. Appl. Biochem., 12, 264 (1990).
16. U. Sprandel and N. Zollner, Res. Exp. Med., 185, 77 (1985).
17. J. DeLoach, S. Peters, O. Pinkord, R. Glew, and G. Ihler, Biochem. Biophys. Acta, 496, 507 (1977).
18. S. K. Jain and S. P. Vyas, J. Microencapsulation., 11, 14, (1994).
19. Y. M. Sayed, M. E. Abdel-Hameed, M. S. Suleiman, and H. M. Najib, J. Pharm. Pharmacol., 40, 727 (1989).
20. P. K. Gupta and C. T. Hung, Int. J. Pharm., 56, 217 (1989).
21. D. A. Lewis and O. H. Alpar, Int. J. Pharm., 22, 137 (1984).