Research Article

Loading Mg^{2+} into Erythrocytes with a Proposed Single Syringe Sterile Loading Process

Ranasinghe K. D. E. P.^{1*}, Thambavita D.², Pathirana W.³

¹Department of Chemistry, Faculty of Science, University of Colombo, Sri Lanka.

²Department of Pharmacology and Pharmacy, Faculty of Medicine, University of Colombo, Sri Lanka.

³Retired Senior Lecturer in Pharmacy, Department of Pharmacology and Pharmacy, Faculty of Medicine, University of Colombo, Sri Lanka.

*Corresponding author: kdepranasinghe@gmail.com

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ABSTRACT

Purpose: Primary hypomagnesemia with secondary hypocalcemia (PHSH) is a genetically defined disease in which there is a malabsorption of magnesium from the intestine. The aim of this research was to encapsulate magnesium into autologous erythrocytes to be used in PHSH patients. A scheme to use a single syringe closed sterile system for the process is proposed. Controlled lysis of erythrocytes and the ability to reseal the loaded erythrocytes were investigated. **Method:** Suitable concentrations of salt solutions required for making reversible pores and resealing of erythrocytes were identified. For making reversible pores, 0.7% NaCl and 2.3% $MgSO_4$ were used separately. A solution of 3.5% $MgSO_4$ was used as the drug loading solution. After the loading step, the isotonicity for the erythrocytes was restored with 0.9% NaCl and 3.0% $MgSO_4$ solutions separately. The ability to load and reseal erythrocytes were assessed by determining the resulting Mg^{2+} concentration through Microwave Plasma Atomic Emission Spectrophotometric (MP-AES) analysis. **Results:** Analysis of results revealed that Mg^{2+} ions can be successfully loaded into erythrocytes through the formation of reversible pores. Resealing failed with 3.0% $MgSO_4$, but the erythrocytes were successfully resealed with 0.9% NaCl solution. **Conclusion:** The need for strictly controlled salt solution concentrations for lysis and resealing were identified. In theoretical concentration determinations, the osmotic coefficient had to be taken into account. The study showed the possibility of loading Mg^{2+} into erythrocytes as a successful drug delivery system.

Key words: Erythrocytes; Primary hypomagnesemia; Secondary hypocalcemia; Magnesium; Osmotic coefficient; Single syringe process

INTRODUCTION

Erythrocyte loaded drug delivery system is being actively investigated currently among several prolonged release systems. The main

advantage is the prospect of prolonged release up to the life span of the erythrocyte which is approximately 120 days.

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In conventional drug delivery systems, the drug is released to the circulation immediately after it is administered, requiring repeated administration. Prolonged release drug delivery systems are innovated to eliminate this cyclical plasma drug concentration levels. Several mechanisms have been successfully launched for the prolongation of oral drug delivery. Membrane controlled, gastric retentive, sustained or controlled release, and self-emulsifying drug delivery systems are some examples.(1) In this study, erythrocyte loading will be investigated to find out if an inorganic drug could be used as a prolonged release drug delivery system.

Blood consists of erythrocytes, that help in the transportation of respiratory gasses. Since 1973, scientists tried to encapsulate mostly the large molecule candidates such as enzymes, peptides, proteins, nucleic acids and genes into erythrocytes and deliver to patients in a more sophisticated way.(2)

Since then erythrocytes have been used as carriers in a wide range of drugs including antineoplastics (e.g. actinomycin D), angiotensin-converting enzyme inhibitor (e.g. enalapril), anti-infective agents (e.g. gentamicin), systemic corticosteroid (dexamethasone), antioxidant drugs, iron chelators, and prodrugs. Similarly, scientists have tried encapsulating several types of enzymes such as alcohol dehydrogenase and aldehyde dehydrogenase, uricase, urokinase, hexokinase and glucose oxidase, through resealed erythrocytes. Anti-HIV peptides, antineoplastic peptides, insulin, erythropoietin, heparin and interleukin 3 like peptides and proteins have been successfully encapsulated into erythrocytes. Different osmosis-based methods have been used in the encapsulation process to open the

erythrocytes and reseal them after the encapsulation. At present, these encapsulated erythrocytes are termed as “golden eggs in novel drug delivery systems” because of their incredible service to the medicinal field.(3)

Advantages of this system include biocompatibility, less side effects, ability to load large amounts of the drug and sustained action.(4)(5)(6) Scientists have used several methods to load suitable candidates into erythrocytes. Among those, there are physical methods (e.g.: electroporation), osmosis-based methods (e.g.: Hypotonic hemolysis, hypotonic dilution, hypotonic pre-swelling), and chemical methods (e.g.: Chemical Perturbation of the Membrane).(5)(6)(7)

Magnesium is available in the human body as Mg^{2+} . This is the second highly available ion in the cells and the fourth highly available ion in the body.(8) Mg^{2+} ions are available in both intracellular and extracellular environments. This ion performs many essential roles in the body. The reference range for serum Mg^{2+} level in a healthy adult is 1.3-2.1 mEqL⁻¹ or 0.65-1.05 mmolL⁻¹.(9) The normal ionized Mg^{2+} concentration inside the oxygenated erythrocytes is about 0.4 mmolL⁻¹.(10)

Magnesium sulfate as a drug is available through the oral route (5 – 10 g in a glass of water) and 20% magnesium sulfate as intravenous injection (0.8 mmol mL⁻¹). Intravenous injections are indicated in hypomagnesemia. It is poorly absorbed from the gut and is excreted mainly by the kidneys. Side effects of magnesium sulfate include nausea, vomiting, thirst, flushing of skin, hypotension, arrhythmia, loss of tendon reflexes, and muscle weakness.(11)

The first primary hypomagnesemia condition was diagnosed in 1965. Later, it has been found that the disease is caused by a recessively inherited gene TRPM6 (9q21.13), which causes intestinal malabsorption of magnesium.(12) This primary condition of disturbance in magnesium homeostasis, secondarily causes hypocalcemia due to impairment of the parathyroid gland and results in generalized tetanic convulsions in the neonatal period.(13)(14) Parathyroid gland produces parathyroid hormone, which helps in calcium homeostasis.(15) Initial therapy for PHS is intravenous or intramuscular magnesium preparations which can adversely affect the site of injection by fungal infections, sometimes, systemic infections. Maintenance therapy usually uses oral administration of high doses of magnesium which may cause gastrointestinal side effects in some patients and hence they require additional parenteral magnesium.(12)

This research study deals with encapsulation of magnesium sulfate by erythrocytes. The primary focus of this study was to find suitable methods to load magnesium ions into erythrocytes with the aim of using in PHS patients in future.

METHODS

All the chemicals and reagents used in the study were of "Analytical grade". Sodium chloride intravenous infusion BP 0.9% w/v (Sichuan Kelun Pharmaceutical Co.Ltd., Chengdu, Sichuan, Lot number: T18105406-2) and dextrose intravenous infusion 5% (B.Braun Medical Industries S/B, 11900, Bayan Lepas, Penang, Malaysia, Lot number: 19015371), were also used. All the glassware utilized were "Class A" glassware. Microwave Plasma Atomic Emission Spectrophotometer (MP-AES) (Agilent

technologies, 4210, United States of America, 2016) was used to analyze the Mg^{2+} concentration of the samples.

Throughout the study three types of solutions (NaCl, $MgSO_4$, and Type 1 Water) were used to suspend erythrocytes. NaCl solutions in different strengths were used because according to literature that is the solution which has widely been used in erythrocyte drugs loading studies. $MgSO_4$ solutions in different strengths were used because the drug of interest is $MgSO_4$ and it is a novel area to explore. Type 1 Water was used as a solvent without ions and as a hypo-osmotic medium.

A stock solution of 1.0% w/v NaCl and a dilution series from 0.3% to 0.8% NaCl were prepared. A stock solution of 4.0% w/v $MgSO_4$ and a dilution series from 2.0% to 3.0% $MgSO_4$ were prepared. The iso-osmolar $MgSO_4$ solutions for NaCl solutions were prepared by considering the osmotic coefficient values as 0.58 for $MgSO_4$ and 0.93 for NaCl.(16) All the centrifugation steps throughout the study were conducted at 1000 g for 3 minutes.(17)

Isolation and purification of erythrocytes from blood: A volume of 1 ml of blood was mixed with a volume of 1 ml ice cold (temperature = $4 \pm 1^\circ C$) 0.9% NaCl and it was centrifuged. The pellet was mixed with 1 ml of ice cold (temperature = $4 \pm 1^\circ C$) 0.9% NaCl and it was centrifuged. This purification step was repeated twice, and 50 μl of the final pellet was observed under the microscope in $\times 400$ magnification.

Determination of the sodium chloride and magnesium sulfate concentrations which reversibly lyses erythrocytes: A volume of 50 μl of the purified pellet was mixed with 1

ml of 0.3% NaCl solution. The same procedure was carried out for the rest of the seven solutions (0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9% and 1.0% of NaCl) and Type 1 Water. After 20 minutes, the solutions (all 9) were centrifuged and carefully observed releasing of hemoglobin to the supernatant. The same procedure was employed for 2.0%, 2.1%, 2.2%, 2.3%, 2.4%, 2.5%, 2.6%, 2.7%, 2.8%, 2.9%, and 3.0% of MgSO₄ solutions.

The NaCl 0.7% and MgSO₄ 2.3% solutions were selected as the highest concentrated solutions which took 15-20 minutes for a controlled hemolysis, judged through careful examination of the rise of red color hemoglobin to the supernatant.

Determination of the intra-erythrocytic physiological magnesium concentration:

This concentration value is critical for other calculations. This step was carried out by suspending erythrocytes in three different media as follows.

- I. A volume of 100 µl of purified erythrocytes was re-suspended in 2.0 ml of MgSO₄ 2.3% solution (label: Mg-E). After 30 minutes, it was centrifuged. The supernatant was analyzed by using the MP-AES for Mg²⁺ concentration. For that, the supernatant was diluted 10000 times by using Type 1 Water and the final solution was filtered through 0.2 µm nylon filters. Two replicates of the sample were prepared.
- II. Same procedure was employed by using a NaCl 0.7% solution for suspending erythrocytes (label: Na-E). The supernatant was diluted only 10 times for the MP-AES analysis.
- III. Type 1 Water was the third medium (label: T1-E). Here again the supernatant was diluted only 10 times.

Determination of the amount of magnesium transferred through intact erythrocyte membrane:

This step was done to determine the Mg²⁺ concentration which penetrates the intact erythrocytic membrane via osmotic mechanism. This finding is essential to study the release kinetics of loaded Mg²⁺ ions. By determining the Mg²⁺ concentration transferred through intact erythrocyte membrane, precautions can be taken during the *in vitro* release kinetic studies. This step was carried out by suspending erythrocytes in two different media as follows.

A volume of 100 µl of purified erythrocytes was re-suspended in 2.0 ml of MgSO₄ 3.0% solution (label: Mg-IE). After 30 minutes, it was centrifuged, and the pellet was re-suspended in 2 ml of MgSO₄ 2.0% solution. After 30 minutes, the suspension was centrifuged, and the supernatant was analyzed by using the MP-AES for Mg²⁺ concentration. For this, the supernatant was diluted 10000 times by using Type 1 Water and the final solution was filtered through 0.2 µm nylon filters. Two replicates of the sample were prepared.

The same procedure was employed by using a NaCl 0.4% solution (label: Na-IE) instead of using the MgSO₄ 2.0% solution. The supernatant was diluted only 10 times.

Determination of the shelf life of erythrocytes in selected media under experimental conditions:

The shelf life of erythrocytes stored in NaCl 0.9%, MgSO₄ 3.0%, dextrose 5%, mixture of 1:1 MgSO₄ 3.0% and dextrose 5% solution and sodium citrate 3.2% solution were determined separately. A volume of 100 µl of the purified pellet was suspended in 2 ml each of the above five different storage solutions. To eliminate microbes freshly boiled and cooled solutions were used to suspend the pellet. The

suspensions were stored in the refrigerator at 4°C. They were centrifuged at weekly intervals. The time taken to lyse the erythrocytes were noted through careful visual examination of the rise of red color hemoglobin to the supernatant.

Loading Mg^{2+} into erythrocytes: A volume of 100 µl of the pellets Mg-E and Na-E derived under subsection “Determination of the amount of magnesium transferred through intact erythrocyte membrane” were separately treated during this step. They were re-suspended in 2.0 ml of $MgSO_4$ 3.5% solution. The solutions were labeled as Mg-LE and Na-LE respectively. After 15 minutes, the suspensions were centrifuged. The pellets were extracted, and the supernatants were analyzed by using MP-AES for Mg^{2+} concentration. Supernatants were diluted 10000 times by using Type 1 Water and the final solutions were filtered through 0.2 µm nylon filters.

Resealing of the reversibly lysed erythrocytes: This was carried out in two different media as follows.

- I. A volume of 100 µl of the pellet of Mg-LE; derived under subsection “Loading Mg^{2+} into erythrocytes” was re-suspended in 3.0 ml of $MgSO_4$ 3% solution. After 15 minutes, the suspension was centrifuged. A volume of 1.5 ml of the supernatant was extracted and it was labeled as Mg-RSE1. This supernatant was analyzed by using MP-AES for Mg^{2+} concentration. During analysis, the supernatant was diluted 10000 times by using Type 1 Water and the final solution was filtered through 0.2 µm nylon filters. After another 15 minutes, the suspension was centrifuged. A volume of 1.5 ml of the supernatant was taken and it was labeled as Mg-RSE2. This supernatant was also analyzed by using MP-AES for Mg^{2+}

concentration by following the same procedure.

The same procedure was employed for the pellets of Na-LE, by using a NaCl 0.9% solution (labels: Na-RSE1 and Na-RSE2) instead of using the $MgSO_4$ 3.0% solution. The supernatant was diluted only 10 times and it was analyzed through MP – AES.

Scheme for single syringe closed sterile drug loading procedure: The drawing of blood, isolation of erythrocytes, washing steps and the loading of the drug must be carried out aseptically with minimum exposure for re-administration of autologous drug loaded erythrocytes into the given patient. The existing procedures entails several high-risk steps in which contamination can result due to frequent transfer of blood and erythrocytes between vessels. To overcome this a single closed container system (sterile syringe) applicable to the entire range of steps involved were identified (Figures 1a-1f). What the figures indicate is the pointing of the needle up or down as and when it must draw in an external medium or expel out a portion from the syringe. In our experience steps requiring separation of erythrocytes in the syringe take a long time and these must be expedited through a suitable centrifugation process. Erythrocyte washing solutions as well as drug solution for erythrocyte loading should be sterile. Several plunger movement steps involved in the process carry a remote potential for contamination. This is an area that needs validation. An alcohol wipe of plunger stem could be suggested as a guard against this.

RESULTS

As expected, an erythrocyte yield of approximately 450 µl/ml of blood was

Figure 1: Schematic diagrams of the proposed steps in single syringe sterile autologous erythrocyte drug loading.

obtained in the isolation process. A photograph of the microscopic field of purified erythrocytes observed under the high power ($\times 400$) of the light microscope is shown in Figure 2. The NaCl 0.7% solution

and MgSO_4 2.3% solution were identified as the highest concentrated solution which took 15-20 minutes for a controlled lysis (Figures 3(a) and 3(b)).

Figure 2 : A photograph of the microscopic field of the isolated erythrocytes under the light microscope ($\times 400$).

Figure 3: Test tube series (a) NaCl and (b) MgSO₄ solutions in determining highest controlled lysis concentration. Lysis concentrations identified were 0.7% for NaCl and 2.3% for MgSO₄. Iso-osmotic concentrations are 0.9% and 3% respectively.

Table 1: Shelf-lives of erythrocytes in five selected media

Solution	Shelf life (days)
0.9% NaCl	< 7
3.0% MgSO ₄	< 6
5% dextrose	< 5
Mixture of 1:1 MgSO ₄ 3.0% and dextrose 5% solution	< 7
3.2% sodium citrate solution	< 7

The results of the experiment carried out to find a suitable solution to store isolated erythrocytes are shown in the Table 1.

Calculations of [Mg²⁺] based on MP-AES analysis results: As shown in the schematic diagrams in Figures 4a-4d erythrocytes were differently treated by suspending in hypotonic, hypertonic and isotonic NaCl and MgSO₄ solutions to that of the intra-erythrocytic environment.

Figure 4: Schematic diagrams showing the direction of movement of the solvent in each step

The MP-AES machine is fed to detect the $[\text{Mg}^{2+}]$ in the solutions. Therefore, when the outer solution is a MgSO_4 solution the MP-AES reading gives the total $[\text{Mg}^{2+}]$ in the outer solution and intra-erythrocyte environment. Since the $[\text{Mg}^{2+}]$ in the outer solution is a known value, $[\text{Mg}^{2+}]$ which contributed in the particular step can be calculated by subtracting outer $[\text{Mg}^{2+}]$ from the MP-AES reading. Here, in some situations a minus sign appears before the calculated value because, the direction of

movement of the solvent along with the solute is the other way around, not in the direction desired. When the outer solution is either NaCl or Type 1 Water, the MP-AES reading can be directly taken as the $[\text{Mg}^{2+}]$ which contributed in the particular step because these two outer solutions do not contain Mg^{2+} ions. The Table 2 shows the average results of MP-AES analysis and the Table 3 shows the calculations done based on the MP-AES analysis results

Table 2: Results of microwave plasma atomic emission spectrophotometric analysis and the average Mg^{2+} concentrations in suspending solvents

Step in which the $[\text{Mg}^{2+}]$ was determined	Label	Replicate 01		Replicate 02		Average $[\text{Mg}^{2+}]$ in the samples (mg dm^{-3})
		Intensity	Concentration (mg dm^{-3})	Intensity	Concentration (mg dm^{-3})	
Intra-erythrocyte $[\text{Mg}^{2+}]$	Mg-E	4.64×10^4	2.87	4.32×10^4	2.67	2.77
	Na-E	4.08×10^4	2.52	4.02×10^4	2.48	2.50
	T1-E	4.94×10^4	3.06	4.17×10^4	2.57	2.82
$[\text{Mg}^{2+}]$ transferred through intact erythrocyte membrane	Mg-IE	3.81×10^3	0.23	3.46×10^3	0.20	0.22
	Na-IE	3.67×10^3	0.22	3.51×10^3	0.20	0.21
$[\text{Mg}^{2+}]$ in loaded erythrocytes	Mg-LE	5.62×10^4	3.48	5.66×10^4	3.50	3.49
	Na-LE	5.62×10^4	3.48	5.67×10^4	3.50	3.49
$[\text{Mg}^{2+}]$ in resealed erythrocytes	Mg-RSE1	3.94×10^4	2.43	3.78×10^4	2.33	2.38
	Mg-RSE2	4.61×10^4	2.85	4.52×10^4	2.80	2.82
	Na-RSE1	2.23×10^6	138	2.19×10^6	136	137
	Na-RSE2	2.23×10^6	138	2.25×10^6	140	139

DISCUSSION

According to the calculations, the intra-erythrocyte $[\text{Mg}^{2+}]$ varies from 25.0 mg dm^{-3} – 28.2 mg dm^{-3} . According to Table 3, the concentration difference when the outer environment is containing a MgSO_4 2.0% solution was a negative value. The explanation for getting a negative concentration difference is the loss of Mg^{2+} ions from the solution. According to the calculation, the concentration of Mg^{2+} ions transferred from MgSO_4 2.0% solution through the erythrocyte membrane is $1.78 \times 10^4 \text{ mg dm}^{-3}$.

During the step done to find the $[\text{Mg}^{2+}]$ in loaded erythrocytes both concentration differences of Mg-LE and Na-LE solutions were negative values. The explanation for getting negative concentration differences is the loss of Mg^{2+} ions from the solutions. According to the calculation shown in the Table 3, for $[\text{Mg}^{2+}]$ in resealed erythrocytes, there was a net flow of Mg^{2+} ions into the erythrocytes from the MgSO_4 solution as the indicated concentration differences were negative values and the concentration values were changing during the two-experimental time period.

However, the $[Mg^{2+}]$ of the NaCl solution had changed significantly. This evidence indicated possible resealing of erythrocytes in the sodium chloride solution.

Table 3: Summary of $[Mg^{2+}]$ calculations based on the microwave plasma atomic emission spectrophotometric analysis results

Step in which the $[Mg^{2+}]$ was determined	Label	Average Mg^{2+} concentration considering the dilution $[C_1]$ ($mg\ dm^{-3}$)	Mg^{2+} concentration in the external environment $[C_2]$ ($mg\ dm^{-3}$)	$[Mg^{2+}]$ in the step specified ($[C_1] - [C_2]$) ($mg\ dm^{-3}$)
Intra-erythrocyte $[Mg^{2+}]$	Mg-E	2.77×10^4	2.3×10^4	$(2.77 \times 10^4 - 2.3 \times 10^4)$ $= 4.7 \times 10^3$
	Na-E	25.0	0.0	25.0
	T1-E	28.2	0.0	28.2
$[Mg^{2+}]$ transferred through intact erythrocyte membrane	Mg-IE	2.2×10^3	2.0×10^4	$(2.2 \times 10^3 - 2.0 \times 10^4)$ $= -1.78 \times 10^4$
	Na-IE	2.1	0.0	2.1
$[Mg^{2+}]$ in loaded erythrocytes	Mg-LE	3.49×10^4	3.5×10^4	$(3.49 \times 10^4 - 3.5 \times 10^4)$ $= -1 \times 10^2$
	Na-LE	34.9	3.5×10^4	$(34.9 - 3.5 \times 10^4)$ $= -3.49 \times 10^4$
$[Mg^{2+}]$ in resealed erythrocytes	Mg-RSE1	2.38×10^4	3.0×10^4	$(2.38 \times 10^4 - 3.0 \times 10^4)$ $= -6.20 \times 10^3$
	Mg-RSE2	2.82×10^4	3.0×10^4	$(2.82 \times 10^4 - 3.0 \times 10^4)$ $= -1.80 \times 10^3$
	Na-RSE1	1.37×10^3	0.0	1.37×10^3
	Na-RSE2	1.39×10^3	0.0	1.39×10^3

Here, $[C_1]$ is the average Mg^{2+} concentration considering the dilution in $mg\ dm^{-3}$ and $[C_2]$ is the Mg^{2+} concentration in the external environment in $mg\ dm^{-3}$.

Based on the results, it can be concluded that the $MgSO_4$ 3.5% solution was successful in loading Mg^{2+} ions into the erythrocytes and the NaCl 0.9% solution successfully resealed the reversible pores. However, $MgSO_4$ solution was not successful as a perforating and resealing solution.

The proposed scheme for single syringe closed sterile drug loading procedure is expected to greatly facilitate the autologous drug loading and administration procedures. The experimental *in vitro* erythrocyte drug loading can now be advanced closer to the *in vivo* human experiments as a result of the

proposed autologous sterile single syringe drug loading procedure (Figures 1a-1f).

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