Available online on www.ijddt.com
International Journal of Drug Delivery Technology 2016; 6(3); 58-63

ISSN: 0975 4415

Research Article

Preparation and *In Vitro* Evaluation of Resealed Erythrocytes as A New Trend in Treatment of Asthma

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Available Online:30th July, 2016

ABSTRACT

Carrier erythrocytes are emerging as one of the most promising biological drug delivery systems investigated in recent decades. Beside its biocompatibility, biodegradability and ability to circulate throughout the body, it has the ability to perform extended release system of the drug for a long period. The ultimate goal of this study is to introduce a new carrier system for Salbutamol, maintaining suitable blood levels for a long time, as atrial to resolve the problems of nocturnal asthma medication Therefore in this work we study the effect of time, temperature as well as concentration on the loading of salbutamol in human erythrocytes to be used as systemic sustained release delivery system for this drug. After the loading process is performed the carrier erythrocytes were physically and cellulary characterized. Also, the in vitro release of salbutamol from carrier erythrocytes was studied over time interval. From the results it was found that, human erythrocytes have been successfully loaded with salbutamol using endocytosis method either at 25 Cº or at 37 Cº. The highest loaded amount was 3.5 mg/ml and 6.5 mg/ml respectively. Moreover, the percent of cells recovery is 90.7± 1.64%. Hematological parameters and osmotic fragility behavior of salbutamol loaded erythrocytes were similar that of native erythrocytes. Scanning electron microscopy demonstrated that the salbutamol loaded cells has moderate change in the morphology. Salbutamol releasing from carrier cell was 43% after 36 hours in phosphate buffer saline. The releasing pattern of the drug from loaded erythrocytes showed initial burst release in the first hour followed by a very slow release, obeying zero order kinetics. It concluded that salbutamol is successfully entrapped into erythrocytes with acceptable loading parameters and moderate morphological changes, this suggesting that erythrocytes can be used as prolonged release carrier for salbutamol.

Keywords: Carrier erythrocytes, asthma, endocytosis, salbutamol, osmotic fragility.

INTRODUCTION

Asthma is an extremely common disorder which affects many people. It is observed as provocative condition with bronchospasm and bronchial hyperactivity[1] Nocturnal asthma, an ailment predominant in two-thirds of the asthmatics, is defined as a inconstant night time exacerbation of the underlying asthma condition accompanying with symptoms exaggeration, augmented airway responsiveness and falling of lung function[2]. Nocturnal asthma is presently controlled by taking either sustained release bronchodilator, or long acting β2 agonists[3,4]. The inability to maintain high blood levels for long period to face nocturnal attack is the major drawback of the existing sustained-release formulations. This may resulted in leaving the patient vulnerable against the worse events of nocturnal asthma[5]. Exploration of advanced ways to administer the presented drugs with conserving its safety and better effectiveness was born due to the high costs of new drug molecule development[6]. Carrier erythrocytes are one of the most promising natural drug delivery systems have been investigated[7]. Resealed erythrocytes are gaining more popularity because of their biocompatibility, biodegradability, ability to circulate throughout the body, ease of preparation and ability to perform extended release system of the drug for a long period[8,9]. They can be used either to target drugs to a specific site in the body, or to provide slow release of encapsulated drugs in the circulation[10]. Drug encapsulation into erythrocyte by endocytosis is more desirable when they used as sustained released carriers, because it has minimal effects on erythrocytes structure and morphology. Certain drugs have been entrapped in erythrocytes by endocytosis, including Vinblastine, Chlorpromazine, Hydrocortisone, Propranolol, Tetracaine, Retinol, and Pravastatin[11]. The introduction of carrier system maintaining suitable blood levels of Salbutamol, as a model drug of β2 agonists, for a long time may resolve the problems of nocturnal asthma medication. A technique that has been proposed for the prolongation of the blood retention time of Salbutamol is their encapsulation in red blood cells (RBCs). Optimally, this extends the blood retention time toward the half-life of RBCs, which is around 30 days in humans[12] and 11 days in mice[13]. In this study the entrapment of Salbutamol by human erythrocytes is accomplished by endocytosis

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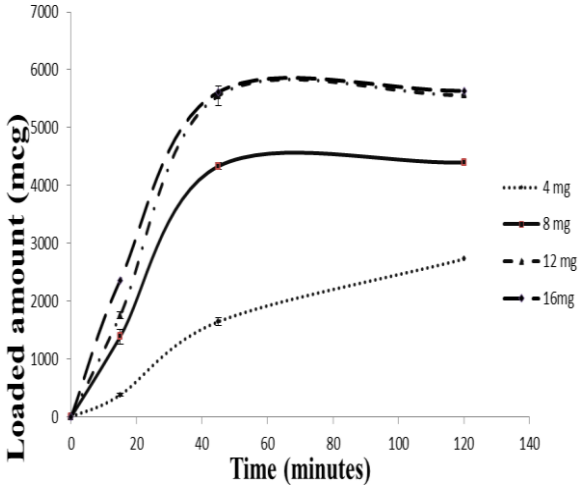

Figure 1: Effect of Salbutamol concentration and incubation time on the amount of Salbutamol loaded on human carrier erythrocytes at 37ºCby endocytosis. Three samples in each group (n = 3).

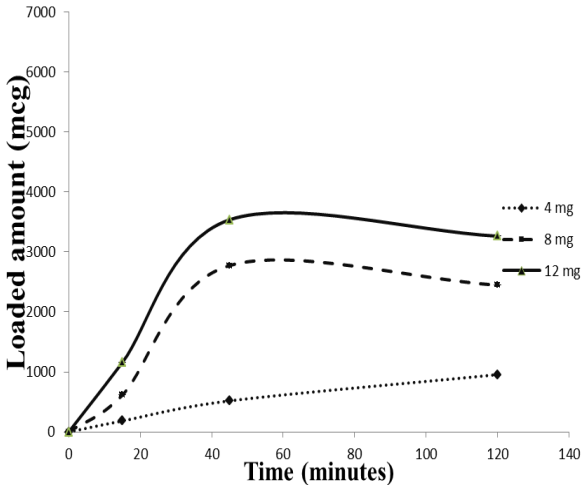

Figure 2: Effect of Salbutamol concentration and incubation time on the amount of Salbutamol loaded on human carrier erythrocytes at 25ºCby endocytosis. Three samples in each group (n= 3).

method. The effect of Salbutamol concentrations and incubation times were studied at different temperatures. The loading parameters, hematological, osmotic fragility of Salbutamol loaded indices as well as erythrocytes were determined.

MATERIALS AND METHODS

Materials

The chemicals used in this study were Salbutamol Sulphate (gift from MUP, Ismailia, Egypt), NaCl (Merck, Germany), KCl, $MgCl_2 \cdot 6H_2O$ (Fluka chemie AGCH), $Na_2HPO_4 \cdot 12H_20$ (BDH-GPR Tm), KH_2PO_4 (Merck, Germany), $MgSO_4.7H_2O$ (Sigma Chemical Co., St. Louis, Mo), adenosine 5-triphosphate (ATP) (Spectrum chemical MFG. CORP), methanol and acetonitrile (HPLC grade) acquired from (BDH). All remaining chemicals were of analytical grade.

Methodology

Salbutamol assay

Spectrofluorometer method (Jasco FP-6200 Spectrofluorometer, equipped with 150 Watt Xenon lamp, Japan) was used for determination of Salbutamol loaded on erythrocytes. Slit widths for both monochromators were set at 10 nm; all measurements were done at medium sensitivity. The difference in fluorescence intensity between the reagent blank and each sample was measured at λ_{em} 611 nm after excitation at λ_{ex} 280 nm[14]. Standard calibration curve was done with linearity range (200 – 5000) monogram / ml with $r^2 = 0.9995$.

Preparation of erythrocyte suspension

The blood specimens were collected by venipuncture from healthy male donor not suffered from acute and chronic diseases into heparinized tubes. Blood samples were centrifuged for 5 min at 5000 rpm. The plasma and the buffy coat were removed by aspiration. Erythrocytes were washed three times in cold phosphate buffer saline (PBS) pH 7.4 with centrifugation for 5 min at 5000 rpm.

Salbutamol loading procedures

The hematocrit of washed erythrocytes was adjusted by PBS to 50%. In 2 ml eppendorff tubes, 400 µl of suspension are added to 400 µl of PBS containing known concentration of Salbutamol and 2.5 mmol of ATP, 2.5 mmol $MgCl_2$ and 2.5 mmol of $CaCl_2$, gently mixing to avoid hemolysis and incubation for 15, 45 and 120 minutes at room temperature. The erythrocytes suspension was centrifuged for 5 min at 5000 rpm and the supernatant is discarded. The packed erythrocytes were washed three times in cold BPS with centrifugation for 5 min at 5000 rpm. Sham encapsulated erythrocytes were also prepared as described without addition of Salbutamol.

Effect of Salbutamol concentration on loading parameters

The effect of Salbutamol concentration on loading efficiency was evaluated using different drug concentrations (4 mg, 8 mg, 12 mg and 16 mg) for all selected incubation times, and the results were compared to obtain the more suitable concentration which gives most appropriate loading parameters.

Study the effect of incubation time

The effect of Salbutamol incubation time on loading parameters was determined for the previous concentrations for different incubation times (15, 45, 120 minutes) and the results were compared.

Study the effect of temperature

Salbutamol loading into erythrocytes was done at 25ºC and 37ºC for the previous different incubation times and concentrations.

Loading parameters

To evaluate the final erythrocyte carriers, three indices were defined as loading parameters:

Loaded amount

The total amount of Salbutamol entrapped in 1 ml of the final packed erythrocytes. The packed erythrocytes were hemolysed by addition of equal amount of distilled water, then addition of methanol for precipitation of protein. The mixture was vortexed for 1 minute, and then, centrifuged at 12000 rpm for 15 minutes. The Salbutamol concentration was measured in the supernatant using spectrofluorometeric assay.

Efficiency of entrapment

Table 1: Effect of Salbutamol incubation time on the percent of Salbutamol loading on human carrier erythrocytes at 37 ^{o}C by endocytosis. Three samples in each group (n = 3)

Drug concentration	Drug incubation times		
(mg/ml)	15 min.	45 min.	120 min.
4	9.49 ± 0.68	41.17 ± 0.54	68.24 ± 1.86
8	17.34 ± 0.45	54.16 ± 1.54	54.96 ± 0.69
12	14.63 ± 0.25	46.28 ± 0.47	44.25 ± 4.97

Table 2: Effect of Salbutamol incubation time on the percent of Salbutamol loading on human carrier erythrocytes at 25 ^{o}C by endocytosis. Three samples in each group (n = 3)

Drug concentration	Drug incubation times		
(µg/ml)	15 min.	45 min.	120 min.
4×10^3	4.56± 0.63	6.47± 2.44	7.99 ± 0.29
8×10^3	15.36± 1.06	34.67 ± 2.73	20.38 ± 1.92
12×10^3	28.94 ± 0.23	44.21± 0.53	27.19± 0.74

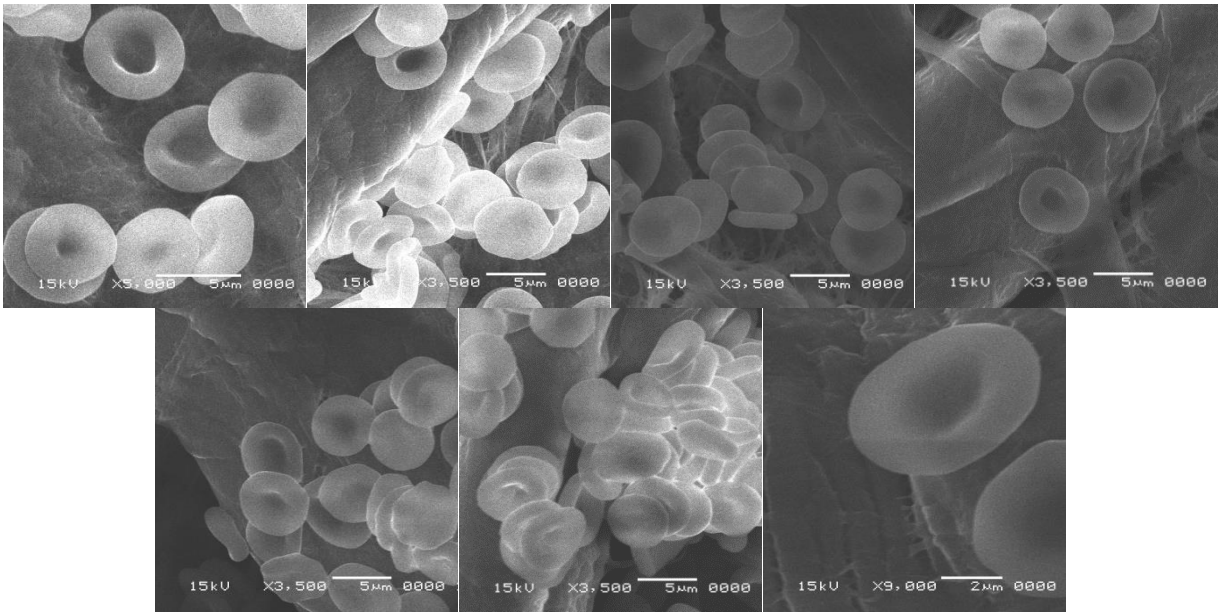

Figure 3: Scanning electron micrograph of Salbutamol loaded erythrocytes by endocytosis at different magnification powers.

The percentage of the loaded amount of Salbutamol measured from the previous step to the total amount of that added during the entire loading process.

Cell recovery

The percentage ratio of the hematocrit value of the final loaded cells to that of the initial packed cells, both measured using equal suspension volumes, and detected using Coulter® AC. T diff TM hematology analyzer (Beckman Coulter, Inc., Brea, CA, USA).

In vitro characterization of Salbutamol loaded erythrocytes

Hematological Indices

To determine the effect of loading process on erythrocytes, normal erythrocytes, erythrocytes suspended in PBS, and Salbutamol loaded erythrocytes were counted. The mean corpuscular volume (MCV: mean cell volume), the mean corpuscular hemoglobin (MCH: average hemoglobin content per each cell), and the mean corpuscular hemoglobin content (MCHC: hemoglobin content per 100 ml of cell volume) were measured using Coulter AC. T diff TM hematology analyzer.

Determination of osmotic fragility behavior of loaded erythrocytes

Erythrocytes resistance against lysis as a result of the osmotic pressure changes of their surrounding media was evaluated. 25µl of erythrocyte sample was added to each of a series of 2.5 ml saline solutions containing 0.0 to 0.8 g% of NaCl. After gentle mixing and standing for 15 min at room temperature, the erythrocyte suspensions were centrifuged at 5000 rpm for 5 min. The absorbance of the supernatant was measured at 540 nm. The absorbance percentage released hemoglobin was expressed as percentage absorbance of each sample in correlation to a completely lysed sample prepared by diluting of packed cells of each type with 1.5 ml of distilled water. Osmotic fragility was studied for each drug concentration.

In vitro releasing study

The release of Salbutamol and hemoglobin from carrier erythrocytes was determined as following; one ml of packed drug-loaded erythrocytes was diluted to 10 ml using PBS the suspension was mixed thoroughly by several gentle inversions. Then, the mixture was divided

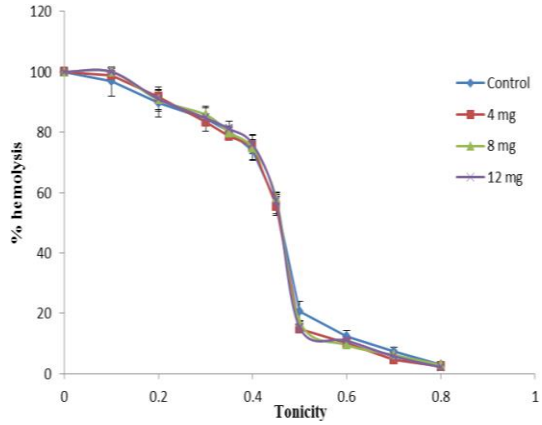

Figure 4: Erythrocyte osmotic fragility of unloaded erythrocytes and erythrocytes loaded with 4, 8 and 12mg/ml Salbutamol. Values are percent hemolysis in corresponding salt concentrations Three samples in each group (n = 3).

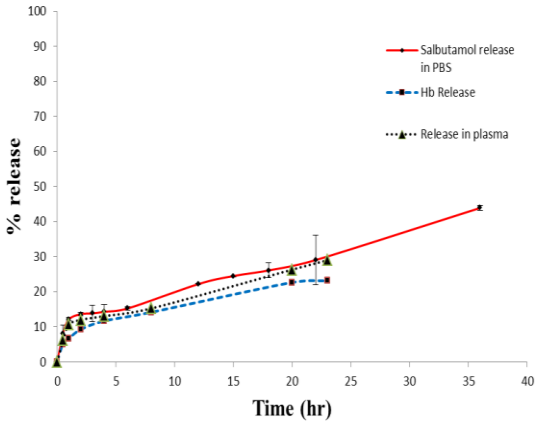

Figure 5: Salbutamol and hemoglobin release behavior in PBS and plasma from Salbutamol loaded erythrocytes. Data is expressed as mean ± SD (n = 3).

into 0.5 ml portions in eppendorff tube. The samples were rotated vertically while incubated at 37°C. At the beginning of the test and also at 0.5, 1, 2, 3, 4,6,12,15,18,22and 36 hours intervals, one of the samples was harvested and then centrifuged at 3000 for 5 min. One hundred µl of the supernatants were separated for Salbutamol assay, and 300 µl for hemoglobin measurement at 540 nm. The release in plasma was also detected with the same procedure.

Scanning electron microscopy (SEM)

The morphological differences between normal and Salbutamol loaded erythrocytes were evaluated using scanning electron microscope (A JEOL JSM-6380, LA., Jeol Ltd., Tokyo, Japan) equipped with a digital camera, at 20 kV accelerating voltage. Both normal and 8 mg/ml Salbutamol-loaded erythrocyte samples were processed as follows. Samples were fixed in buffered glutaraldehyde. The samples were rinsed 3 times for 5 min in phosphate buffer and then, fixed in osmium tetroxide for1hr. The samples were then rinsed with distilled water and dehydrated using a graded ethanol series: 25, 50, 75, 100, and another 100%, each for 10 min. The samples were rinsed in water, removed, mounted on studs, sputter-coated with gold, and then viewed using SEM.

Statistical analysis

The statistical differences between native and loaded erythrocytes were analyzed by one way ANOVA followed by the Bonferroni multiple comparison test, using IBM SPSS Statistics 19 Software, (SPSS Software, Inc.). The results with $p<0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Encapsulation of Salbutamol in human erythrocytes

The effect of time, temperature as well as Salbutamol concentration on the loading process of Salbutamol into human erythrocytes by endocytosis method was studied, as trial to obtain prolonged release system. The highest level of Salbutamol loaded into erythrocytes was achieved using 12 mg/ml of the drug, at 37ºC and 45 minutes incubation time. The loading of Salbutamol into erythrocytes was directly proportional to increase in Salbutamol concentration in the incubation medium, from 2-16 mg/ml concentration range till reach 5.5 mg/ml of erythrocytes. This can ensure sufficient entry of Salbutamol into the body upon reinjection of fairly low volumes of the packed cells, because the dose of the dose of Salbutamol injection is only 0.1 mg. This result confirmed that temperature increase resulted in increase in the amount of Salbutamol loaded into erythrocytes which is in agreement with previous studies[15]. The cell membrane activity was increased till reach optimum temperature 37ºC, This result could be attributed to the presence of calcium ions as well as ATP which stimulates the endocytosis of Salbutamol by erythrocytes. This is supported by the observation Harisa and his workers (2011) which reported that the calcium ions and energy source (ATP) stimulate drug uptake by erythrocytes through membrane invagination and formation of endocytotic vacuole. The drugs induced endocytosis is dependent on the persistence of erythrocyte energy sources[16].

Loading parameters

Loaded amount

The loaded amounts of Salbutamol at 25ºC and 37ºC were determined; at 25 ºC the highest loaded amount was 3.5 mg while it was5.5mg at 37 ºC as shown in figures (1-2). Other polar drugs gave nearly similar results like pravastatin[17] and dexamethasone[18]. These loaded amounts are suitable to Salbutamol dosing upon reinjection of low volumes of drug loaded erythrocytes to the host body.

Loading efficiency

The efficiency of drug loading at 25ºC and at 37ºCwas affected by concentration and incubation time as shown in tables (1-2). It was started from 4.6% after incubation for 15 minutes using drug concentration 4 mg at 25ºC, and increase upon increasing concentration. The higher percent was 44.2% that given at 12 mg after 45 minutes. In comparison tothe results obtained at 37ºC, it was found to be higher at 37ºC. The loading efficiency reaches 68.2% at 4mg after 120 minutes; while it decreased upon increasing concentration. The augment in the loaded amount is depending on the fact that the normal biological temperature enhances the cell biological functions including endocytosis.

Cell recovery

A cell recovery of Salbutamol loaded erythrocytes was 90.7± 1.64%, which proved that the loading process and the drug have a quite effect on the erythrocytes. This is practically better than the recovery results reported in other studies such as Paclitaxel and phenytoin[19,20] due to the injurious effect of drug or loading process respectively.

In vitro characterization of Salbutamol loaded erythrocytes

Hematological Indices

MCV, MCH and MCHC were characterized to determine the impact of the encapsulation process on the hematological properties of the erythrocytes[21].These parameters may offer some valuable evaluations of the biological state of Salbutamol loaded erythrocytes. The mean hematological parameters of the Salbutamol loaded erythrocytes obtained with different concentrations, control cells and sham encapsulated cells (after loading process but without using drug) are represented in table (3). The non-significant change in MCV is observed except at higher concentration12 mg ($P < 0.05$). These records proved that Salbutamol loading mechanism into erythrocytes occurs either by encapsulation or binding to the cell membrane[22]. Non-significant change in both MCH and MCHC ($P < 0.05$) is supported by SEM results and osmotic fragility data that will discussed later, which can be explained by neither Salbutamol nor loading process has damaging effect on a physical and/or functional barrier of erythrocyte, that prevent hemoglobin loss from carrier erythrocytes. These findings proved that erythrocytes can preserve its morphological and functional characters after Salbutamol loading, which make it able to evade from macrophage after reinjection. This ability allows the loaded erythrocytes to circulate through the body like the natural one with long life span.

Osmotic fragility behavior of Salbutamol loaded erythrocytes

The susceptibility of erythrocytes to osmotic lysis with respect to serial dilution of NaCl (0.0- 0.8%) is determined by meaning of osmotic fragility which acts as indicator of the possible cell membrane integrity changes. There is no significant difference in the osmotic fragility of loaded erythrocyte at 4, 8, 12 mg/ml Salbutamol when compared to that of unloaded and sham-encapsulated erythrocytes (figure 3). The osmotic fragility indexes were found to be the same (0.45% NaCl concentration), proving that the loading process at these concentrations has minimal effect on the wall integrity. Several studies reported the effect of drugs on fragility behavior of erythrocytes. Hamidi and his workers stated that osmotic fragility of loaded erythrocytes is lower than unloaded cells[23]. On contrast, another study revealed that osmotic fragility of carrier erythrocytes is higher than unloaded cells[24]. In The present study, the behavior of Salbutamol loaded erythrocytes towards serial concentration of NaCl (0.0- 0.8%) is similar to native unloaded cells, which acts as an indicative of more homogeneous cell population that increase the possibility of loaded erythrocytes the body like normal one giving opportunity to in vivo sustained release for several days.

Scanning electron microscopy

Erythrocytes loaded drug by endocytosis methods were scanned by scanning electron microscopy at different magnifications as shown in figure (4). Different stages of normal biconcave shape and minimal changes in morphology are resulted. This result showed that the loading process and/or the drug have no deleterious effects on erythrocyte shape keeping it similar to native cells, which gives the opportunity for carrier erythrocytes to life span like native[25]. The unaffected erythrocyte shape and topology proved in this study, being one of the main factors in erythrocyte kinetics in circulation, could be potentially beneficial in terms of a successful long circulating carrier preparation. This result recommended that loaded erythrocytes can be used for sustained release of Salbutamol.

Salbutamol release

The in vitro release profile of Salbutamol from carrier erythrocytes in PBS and plasma at 37ºC is shown in figure (5) As seen, the Salbutamol efflux from carrier cells showed rapid release of 12% of drug within first 1hour, indicating that, the percent of drug which was released quickly may be bound on to the surface of the membrane. After this, there was a very slow release of the drug which reaches 43% after36 hours, obeying zero order kinetics with coefficient of correlation (R^2) equal 0.925. Hemoglobin release pattern from loaded cell is comparable to Salbutamol release, confirming that the release mechanism of Salbutamol occurred by cell rupture. Like other polar drugs, Amikacin sulphate[26] and Bovine serum albumin[25], there will be sustained release of the drug as it follows zero order kinetics that depends on cell membrane rupture as they can't diffuse through the membrane. Thus, carrier erythrocytes may be a good candidate for Salbutamol sustained release for nearly 4 days, which make it possible to be used in treatment of asthmatic patient.

CONCLUSION

This study reports the feasibility of erythrocytes as a carrier for Salbutamol. Salbutamol was loaded successfully on human erythrocytes with acceptable loading parameters using endocytosis method. Salbutamol loaded erythrocytes have morphology and fragility like natives, proving that both Salbutamol and endocytosis are less destructive to erythrocytes and preserve the cells fragility and morphology. Salbutamol release from loaded erythrocytes obeying zero order kinetics and may persist in the body for more than 4 days. Release results can be considered a measure of success for using in treatment of nocturnal asthma. To prove this, *in vivo* drug delivery efficacy of these cellular carriers needs to be evaluated during the future in vivo studies.

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