Original Article

DEVELOPMENT AND *IN VITRO* EVALUATION OF VALSARTAN-LOADED RESEALED ERYTHROCYTES

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ABSTRACT

Objective: Resealed erythrocyte technique has attempts for preparation of valsartan loaded reservoir type using red blood cell as a carrier.

Methods: The resealed erythrocytes were prepared by using glutaraldehyde as a cross-linking agent by Preswell dilution technique and sodium chloride as the medium has been made to predict mechanism of drug release and absorption based on the order of release. The prepared resealed erythrocytes was characterised as a percentage of cell recovery drug content, osmotic shock, turbulence fragility, osmotic fragility, *in vitro* drug release studies, and hemoglobin content study.

Results: The result of the study showed that the resealed erythrocytes prepared with sodium chloride (9 %) showed biconcave shape, an assay of 6.5±0.4 %, osmotic shock (0.028±0.004 μg/ml), turbulence fragility of 0.228±0.046 μg/ml, and 44.38±5.54 % drug release in 8 h. The drug release kinetics was studied and found that release from spherical matrices, first-order model with non-fickian diffusion with and the dissolution occurs in planes that are parallel to the drug surface pattern.

Conclusion: VaL administration could be avoided the number of drawbacks associated with systemic delivery and perhaps maintain a relatively constant plasma level during a lengthy course of treatment.

Keywords: Resealed erythrocytes, Valsartan, Cross-linking, Osmotic fragility

INTRODUCTION

There are now thirty primary drug delivery products available in the market with an annual growth rate of 15%, the combined yearly income for all of these are around US$33 billion (based on global product revenue). The requirement for safe drugs that can reach their intended targets and have fewer adverse effects is one of the factors driving the rise in interest in drug delivery. In reality, the bio-distribution of drugs throughout the body is precisely the causes the majority of issues with systemic drug administration [1].

A perfect drug has been ideally exhibit its pharmacological activity solely at the target site, at the lowest effective concentration, and without having any detrimental effects on compartments that are not the target site. The currently known delivery technologies use carriers that are either more complicated multicomponent complexes or simple, soluble macromolecules (such as soluble synthetic polymers, monoclonal antibodies, and particulate biodegradable polymers polysaccharides) (microparticles, microcapsules, cell sparks, cells, liposomes, lipoproteins, and erythrocytes) [2].

Unfortunately, there are times when the body perceives the drug targeting mechanism as non-self, and unanticipated toxicities may prevent the drug from working as intended. This is the situation with the initial generation of monoclonal antibodies that were combined with cytotoxic drugs or other soluble carriers in preclinical experiments. The prepared of self-emulsified, computer-controlled pharmacyte medical nanorobot systems, which can deliver pharmaceutical drugs to particular body targets at predetermined time intervals, has been proposed as the ideal drug delivery method [3].

Although the ideal drug delivery system has not yet been developed, traditional drug formulations have made tremendous advances in recent years. Among all the cell-based delivery methods, some of them are believe that the above-mentioned ideal drug delivery system is the closest to it. Transduced cells with the ability to express pharmaceutically relevant substances and cell carriers with the potential to be loaded with drugs or treatments might be divided into two groups. The carrier cells in this group might disperse the drug content throughout the body, at specific locations, or by directing the drug to other pertinent cells [4].

Blood can be isolated by being removed via a heart or splenic puncture (for small animals) or through veins (for bigger animals) and placed in a syringe with an anticoagulant drop. The entire blood was spun in a chilled centrifuge at 250.400 rpm for 5 min at 4±1 °C. The packed cells are carefully stripped of the serum and buffy coatings, and then they are washed three times in phosphate buffer saline (PBS, 7.4 pH). The washed erythrocytes are then diluted in PBS and kept at 4 °C until they are needed [5].

The most numerous cells in the human body are erythrocytes, which make up 5.4 million cells per mm^3 of blood in a healthy male and 4.8 million cells per mm^3 in a healthy female. Dutch scientist Lee Van Hock first identified these cells in blood samples from people in 1674. Hope Seyler discovered haemoglobin and its critical function in delivering oxygen to diverse bodily areas in the 19th century Biconcave discs; erythrocytes have a volume of 85-91 m^3, an average diameter of 7.8 m, a thickness of 2.5 m at the perimeter, and a thickness of 1 μm in the middle [6].

Erythrocytes may fit through capillaries as small as 3 μm in width thanks to their flexible, biconcave shape. The structure of mature erythrocytes is rather straightforward. They lack organelles like a nucleus. Heme-containing protein haemoglobin, which is responsible for O_2-CO_2 binding inside the erythrocytes, is enclosed by their plasma membrane. Erythrocytes' primary function is to carry oxygen from the lungs to tissues and the CO_2 those tissues create back to the lungs. Erythrocytes are the body's highly specialised O_2 delivery mechanism as a result [7]. The absence of a nucleus leaves all intracellular space open for the transfer of oxygen. Furthermore, erythrocytes do not utilise any of the oxygen they are transporting since they lack mitochondria and produce energy anaerobically.

A high level of biocompatibility, especially when autologous cells are employed to load drugs, total biodegradability, and the absence of

hazardous products as a result of carrier biodegradation preventing any unwanted immunological reactions to the drug that is encapsulated, significant protection of the organism from the drugs hazardous side effects, such as antineoplastics, the circulating carrier erythrocytes have an incredibly longer lifespan than the synthetic carriers [8]. The life-span of the generated carrier cells may be equivalent to that of the regular erythrocytes under ideal loading process conditions, a readily adjustable lifetime that can range from a few seconds to several months, desirable size range and size and shape that are very homogeneous. Protection against endogenous factors inactivating the loaded chemical.

Possibility of specific drug delivery to the relatively inert intracellular environment of the RES organs. A vast range of substances having the potential to be entrapped inside the erythrocytes, as well as the availability of expertise, procedures, and facilities for handling, transfusing, and dealing with erythrocytes. Possibility of loading a reasonably large dosage of the drug into a tiny volume of erythrocytes, ensuring that the dose is sufficient in both human and animal trials employing a small volume of erythrocyte samples.

Valsartan (VaL) is a type I (AT1) angiotensin receptor-selective angiotensin II receptor antagonist (also known as an ARB, or angiotensin receptor blocker). VaL is primarily used to treat congestive heart failure, excessive blood pressure and to prolong life after a heart attack [9]. In order to lower blood pressure, VaL inhibits the activities of angiotensin II, which include constriction of blood vessels and activation of aldosterone. The drug forms an antagonistic bond with angiotensin type I receptors (AT1). Compared to ACE inhibitor drugs, which prevent the conversion of angiotensin I to angiotensin II, this mechanism of action is distinct. Given that VaL operates at the receptor and because angiotensin II is produced by several enzymes in addition to ACE, it can give a more thorough antagonism of the peptide. Additionally, unlike ACE inhibitors, VaL has no impact on the metabolism of bradykinin.

MATERIALS AND METHODS

Materials

Valsartan (VaL; purity>99.97 %) was a gift sample received from FDC limited Mumbai, India. Methanol, ethanol, and isopropyl alcohol were purchased from Thermo Fisher scientific Pvt. Ltd. Mumbai, India. Used chemicals and reagents were of analytical grade in this research.

Collection and storage of blood

Fresh whole blood was obtained from a heart or splenic puncture into heparinized tubes by venipuncture, promptly frozen to 4 °C, and kept in storage for no more than two days. The preparation and evaluation of resealed erythrocytes steps by step are shown in fig. 1.

Preparation of drug-loaded erythrocytes

Following aspiration of the buffy coats and centrifugation of the erythrocytes from serum coats for 5 min at 2500 rpm at 4 °C, the buffy coats are then washed three times with a cold, 7.4-pH phosphate buffer solution (PBS). The cleaned cells are suspended in buffer solutions according to the required hematocrit levels, and they are frequently kept in acid-citrate dextrose buffer at 4 °C for up to 48 h before use [10].

Preparation of resealed erythrocytes

Preswell dilution technique

A flask holding a 50 percent v/v suspension of RBCs (erythrocytes) up to the point of haemolysis was filled with a solution that was hypotonic (0.3 percent w/v NaCl solution). 10 ml of a 1 percent w/v drug solution was added to the swollen RBCs, and the isotonicity of the swollen erythrocytes was maintained by adding a hypertonic solution (1.3 w/v NaCl solution) to reseal the membrane. This process was then gently centrifuged to remove any unentrapped drug solution on the membrane surface [11]. The suspension was cleaned three times with pH 7.4 phosphate buffer saline (PBS), then appropriately diluted with PBS and refrigerated at 4 °C. Erythrocytes

were created utilising the aforementioned technique using a 1 percent solution of various crosslinking agents, such as glutaraldehyde.

Evaluation of released erythrocytes

Preparation of stock solution

100 mg of VaL was dissolved in methanol (30 ml) and then made up to a volume of 100 ml with methanol. From the stock solution, 1 ml was diluted to 100 ml with methanol to form the sub-stock solution then 2 to 20 ml of solutions were drawn and made upto to obtain a concentration range of 2 to 12 μg/ml [12]. In a 1.0 cm cell with a solvent blank, a standard VaL solution with a concentration of 10 μg/ml was scanned in the UV region (400-200 nm), and zero-order and second-order spectra were acquired. The absorbance was measured at 250.40 nm.

Calibration curve

The stock solution was properly diluted in methanol to create the working solution (30 μg/ml). To achieve a concentration in the range of 10–50 μg/ml, aliquots of VaL stock solution were transferred into a succession of 25 ml volumetric flasks up to the mark with methanol. At 250.40 nm, the absorbances of each of the ensuing solutions were measured in comparison to solvent blanks. With a correlation value of 0.992, the calibration curve was shown as concentration versus absorbance throughout the range of 2–12 μg/ml.

Studying morphology and percentage of cell recovery

The amount of intact cells per cubic millimetre of packed erythrocytes before and after the drug loading can be used to calculate the percentage of cell recovery. Erythrocytes that are normal or drug-loaded can be examined using phase contrast or electron microscope [13].

Drug content

In this method, 1 ml of drug-loaded resealed erythrocytes (suspensions) were diluted using 10 ml double distilled water, boiled for 20 min at 50-60 °C and filtered through 0.22 μm filters. Drug content in the supernatant is estimated spectrophotometrically at 250.40 nm [14].

Osmotic shock

Osmotic shock is the term used to describe the abrupt exposure of drug-loaded erythrocytes to an environment that is not isotonic in order to test the resilience of sealed erythrocytes to the stress and retain both their structural integrity and appearance. Incubating the 1 ml of VaL loaded resealed erythrocytes suspension (10-50% hemotocrit) with 10 ml of distilled water for 15 min followed by proper mixing of the suspension at low speeds and centrifugation. The supernatant of the drug content was estimated spectrophotometrically [15].

Turbulence fragility

The impact of injection pressure and shear stress on the integrity of the loaded cells in resealed erythrocyte formulations. 1 ml of VaL loaded erythrocytes was suspended in 10 ml of PBS buffer solution and stirred at high speeds for 2 h. The supernatant of the drug content was estimated spectrophotometrically [15].

Osmotic fragility

1 ml of VaL-loaded resealed erythrocyte suspension is suspended in 10 ml of isotonic saline and incubated separately in a stepwise increasing concentration of sodium chloride solution (0.9-1 % w/v) at 37 °C for 10 min [15]. Following this, the supernatant is centrifuged at 25000-3000 rpm for 10 min, and the drug content was checked in the supernatant. It is based on the fact that cells become less resistant to hemolysis as hypotonic saline concentration decreases.

Drug release studies

To exploit the release kinetics of drug from carrier erythrocytes, 1 ml erythrocyte suspension was packed in a dialysis bag (12500 M.

W) and suspended in 100 ml of PBS buffer solution. The solution in receptor compartment is stirred continuously using magnetic stirrer [16]. The procedure was carried out for 24 h. Samples of 5 ml volume were withdrawn for every one-hour maintaining sink conditions and drug content in the aliquot was estimated spectrophotometrically.

In vitro drug release and hemoglobin content study

Periodically, the release of haemoglobin and drugs *in vitro,* drug-loaded cells were stored in amber-colored glass containers, the cell suspension (5 percent hematocrit in PBS) is kept at 40 °C.

Periodically, the clear supernatant is removed using a hypodermic syringe fitted with a 0.45 filter, deproteinized with methanol, and its drug concentration was measured. After centrifugation, the supernatant from each sample is collected and tested for haemoglobin release using the procedure in the following formula [10].

$$\% \text{ hemoglobin release} = \frac{\text{A540 of sample} - \text{A540 of background}}{\text{A540 of 100 \% hemoglobin}} \times 100$$

A_{540} stands for absorbance at 540 nm.

Fig. 1: Various process of the preparation of resealed erythrocytes (a) centrifugation, (b) magnetically stirring, (c) mixing, (d) turbulation, and (e) release study

RESULTS AND DISCUSSION

The VaL drug contents were estimated by measuring the absorbance at 250.40 nm. The VaL drug was found to be soluble in methanol and ethanol. The standard calibration curve for VaL was prepared in methanol as shown in fig. 2a. This approach agreed with BP requirements and Beer's law in the concentration range of 2 to 12 µg/ml, demonstrating the VaL purity (fig. 2b).
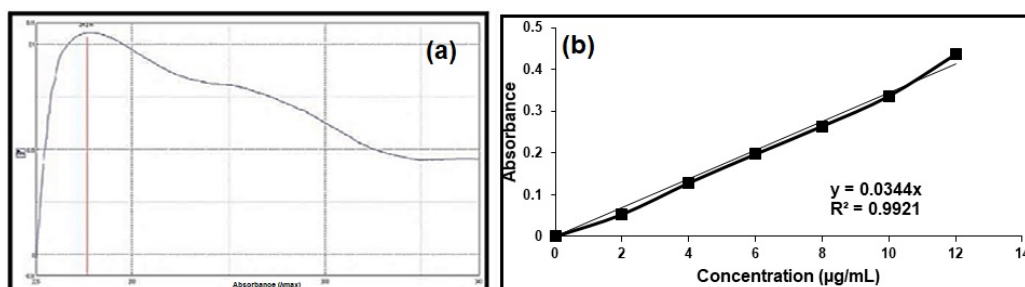
**Fig. 2: (a) Standard spectra and (b) calibration curve of VaL**

Characterization of resealed erythrocytes

The cells showed in fig. 3, exhibits the slight change in their biconcave shape due to drug loaded in the corpuscles and cells in form of spherostomatocytes (uniconcave) existing with pherocytes. The cell membrane invaginations indicated that cells undergo morphological changes during drug loading [17].

Drug content

Drug content in all the formulations was found to be ranging from 40–65 % due to the method used to load the drug (press wall method) compared with other methods. The percentage drug released during osmotic shock study was found to be ranging from 0.40-0.43% showed least drug release indicating that the formulation was resistant to the osmotic shock produced by the changes in the osmotic conditions [18]. The percentage drug releases during turbulence fragility shock around 3.5 clearly indicate that the shear force and pressure involved to fragile the membranes during centrifugation are shown in the table 1.
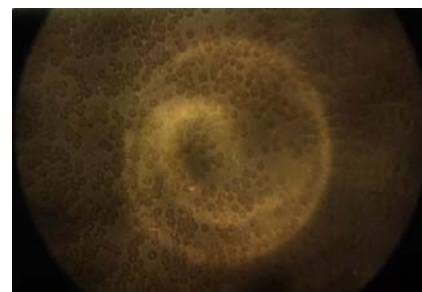
Fig. 3: Photograph of drug-loaded erythrocytes

Table 1: Evaluation parameters of assay, osmotic shock and turbulence fragility studies

S. No.	Evaluation parameters	Amount of VaL released (µg/ml)	Amount of VaL released (µg/100 ml of dilution)	mg/ml of stock solution	% Drug content
1	Drug content study/Assay	6.5±0.4	657±40.6	0.65±0.05	65.27±3.67
2	Osmotic shock study	0.028±0.004	2.85±0.42	0.002±0.001	0.43±0.04
3	Turbulence fragility study	0.228±0.046	22.8±0.46	0.022±0.002	3.5±0.84

Each value represents mean, *n* = 3±SD.

Osmotic fragility

The percentage of drug release was found to be increasing as the concentration of sodium chloride was increasing in all the preparations. The increase in the concentration of sodium chloride leads to hyper tonicity which in turn leads to the drug release due to shrinkage of the cells. The percentage drug release at 1.0 NaCl concentration was found to be ranging from 23-55% (table 2).

Table 2: Osmotic fragility study

S. No.	Nacl conc.	Amount of VaL released (mcg/ml)	Amount of VaL released (Mcg/100 ml of dilution)	mg/ml of stock solution	% Drug content
1	0.9	0.05±0.002	5.2±0.2	0.005	0.80±0.09
2	0.92	0.25±0.03	25.4±3.0	0.025	3.90±0.03
3	0.94	0.45±0.05	45.5±4.14	0.045	7.0±0.81
4	0.96	0.72±0.09	72.2±9.4	0.072	11.1±1.53
5	0.98	1.11±0.04	111.0±3.3	0.111	17.1±1.07
6	1	1.55±0.08	155.0±8.2	0.155	23.9±1.45

Each value represents mean, $n = 3\pm SD$.

In vitro VaL release

The *in vitro* drug release was found to be linear in all the formulations. The drug release percentages in the first 6 h were found to be ranging from 44-55% showed fig. 4a as the sustained release. The *in vitro* drug release first-order kinetics and the regression values were found to be above 0.99 [19]. The data was subjected to Higuchi's diffusion, the regression values of all the formulations was found to be above 0.92. The plots were fairly linear and we can conclude that all the formulations follow Higuchi's diffusion. The data was subjected to the Korsmeyer-Peppas model, the slope values are ranging from 0.5-1, indicating that the diffusion is Non-Fickian diffusion [20].

Release kinetics

The mechanism of drug release was explored by subjecting the data to kinetic analysis by fitting to various mathematical equations and models viz., zero-order (fig. 4b), first order (fig. 4c), Higuchi (fig. 4d), Korsmeyer-Peppas (fig. 4e), and Hixon crowell (fig. 4f) models (table 3). On the basis of higher regression values obtained, the VaL resealed erythrocytes followed first-order kinetic (R^2 = 0.992) and followed Korsmeyer-Pepas equation (R^2 = 0.9922) and the dissolution occurs in planes that are parallel to the drug surface pattern [21].

Erythrocytes (RBCs) have received a great deal of attention as a potentially effective carrier system for the transport of active compounds. Resealed RBCs are preferable to other carriers because they have several exceptional qualities, such as great biocompatibility and biodegradability, a prolonged half-life in circulation, and a good ability to entrap a variety of medications [1]. From this chapter offers an in-depth understanding of different drug-loading methods, RBC characterization, and biological applications. [22].

Table 3: Calculated drug release kinetics

Time	Square root of time	Log time	Cumulative VaL released	Cumulative percent VaL released	Cumulative percent VaL remaining	Log cumulative percent VaL released	Log cumulative percent VaL remaining	Cube root of % VaL released
1	1.0	0	0.011	2.8	97.2	0.44	1.98	1.41
2	1.41	0.30	0.028	7.8	92.2	0.89	1.96	1.98
4	2.0	0.60	0.041	14.2	85.8	1.15	1.93	2.42
6	2.44	0.77	0.082	35.0	65.0	1.54	1.81	3.27
8	0.90	0.90	0.027	54	46.0	1.732	1.66	3.8

As a result, it suggests a link between the diffusion and erosion mechanism, or "anomalous diffusion," and may also suggest that the drug release is regulated by many processes. The plot was linear; the linear regression coefficient value is 0.998. This indicates that considerable erosion of the tablet has taken place. During the release of drug from the erythrocyte membrane has eroded.
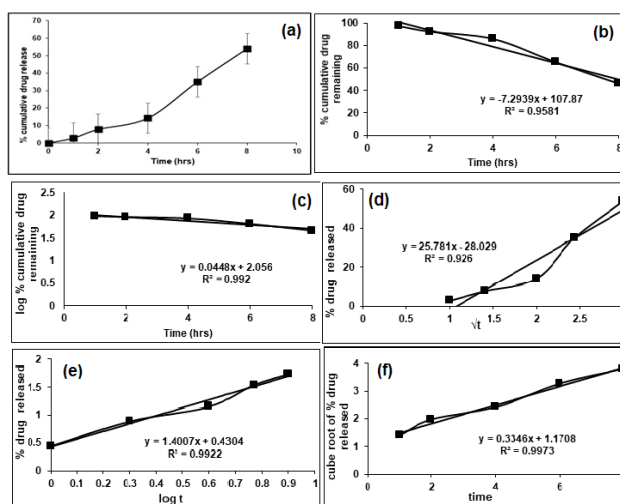
Fig. 4: (a) *In vitro* drug release profile, (b) zero order, (c) first order (d) Higuchi, (e) Korsmeyer-Pepas, and (f) Hixcon crowel kinetic release model

The value of the linear regression was computed and found to be 0.958. Therefore, it is evident that the prolonged drug release and did not follow zero-order kinetics. The value of the linear regression was computed and found to be 0.992. Therefore, it is evident that the prolonged-release tablet's drug release adheres to first-order kinetics. The linear regression coefficient value is 0.998 and the plot was linear. It follows that the drug release complies with the diffusion mechanism. 0.614 was the release exponent.

Resealed erythrocytes may be loaded with a range of active medicinal compounds and are biocompatible, biodegradable, have a long circulation half-life, and are biocompatible. Re-sealed erythrocytes are preferable to other drug delivery methods because they have a number of benefits over them. By taking a blood sample from the target organism and isolating the erythrocytes from the plasma, carrier erythrocytes are prepared [23]. The cells are split using a variety of techniques, the drug is then trapped inside the erythrocytes, and then the carriers are resealed and given the name "resealed erythrocytes."

CONCLUSION

The conclusions drawn from the present study reveals the VaL resealed erythrocytes were successfully prepared using glutaraldehyde using any cross-linking agent by using the Preswell dilution technique. Resealed erythrocytes was evaluated for the parameters like drug assay, osmotic shock, turbulence fragility and osmotic fragility and *in vitro* drug release studies. Resealed erythrocytes have been proposed for use in a variety of applications during the last ten years, including drug delivery, enzyme replacement treatment, and more. For a secure and reliable administration of diverse drugs for passive and active targeting, the use of resealed erythrocytes appears promising.

AUTHORS CONTRIBUTIONS

All the authors are contributed equally.

CONFLICTS OF INTERESTS

The authors declare that there are no conflicts of interest.

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