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LOADING INSULIN INTO HUMAN ERYTHROCYTES AND DETERMINING THE INTRAERYTHROCYTIC INSULIN CONCENTRATION AND STABILITY

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ABSTRACT

The research focuses on encapsulating insulin within autologous erythrocytes to assess the stability of this novel sustained-release drug delivery system. A secondary objective involves proposing a computerized application to streamline the analysis of intraerythrocyte insulin concentrations using UV absorbance measurements. Isolated erythrocytes were treated with concentration series of NaCl to identify the optimum concentration of NaCl for reversible pores. Insulin was then loaded to the cells under simple diffusion. The erythrocytes were resealed with 0.9% NaCl and the stability period of insulin-loaded erythrocytes was studied. A calibration curve of insulin was developed using UV absorption data from a standard dilution series of soluble insulin. The data was used to develop a computerized application to analyze loaded soluble insulin in to erythrocytes. The soluble insulin showed good liner agreement in the range of 0.2 to 0.8 absorbance at 276 nm ($r=0.998$). The optimum concentration of NaCl solution was 0.7% w/v to effect limited lysis of the erythrocytes. The optimum concentration of soluble insulin was 20 IU/mL to load into the erythrocytes. An insulin amount of 6.97±0.17 IU, 7.28± 0.24 IU, and 7.69±0.49 IU in 10, 20, and 30 minutes of simple diffusion time ($p>0.05$) had loaded into 1.00 mL of erythrocytes. The stability period for insulin loaded erythrocytes was 72 hours. The 0.7% w/v NaCl solution effectively creates reversible pores on erythrocyte membranes for loading insulin. The hypo-osmolarity based insulin loading process showed the possibility of loading insulin into erythrocytes and using it as a novel insulin delivery system.

KEYWORDS: Insulin determining computer application, Intra-erythrocyte insulin stability, Resealed erythrocytes.

INTRODUCTION

The most popular way to administer insulin is through subcutaneous injections, which might cause pain and discomfort even when they are effective. Additionally, regular insulin administration affects the quality of life of patients, and the patients or caregivers should make the extra effort to keep the insulin under proper storage conditions throughout the lifelong treatment.

"Insulin-loaded erythrocytes" is a novel drug delivery system that can overcome most of the disadvantages of traditional insulin delivery systems. The 120-day life span of erythrocytes is used in this sustained release effort, where insulin will be released gradually as the older erythrocytes break down. Autologous erythrocytes are degradable. non immunogenic and biocompatible. They show movement across the whole circulatory system and defense against early degradation, inactivation, and excretion from the body with less side effects.[1]

Bioactive substances, including drugs, may be transported by erythrocytes in a way that is compatible with their internal environments. For peptides, enzymes, and medications, these work well as biological carriers. Nowadays, the best yield of medication encapsulation in erythrocytes is achievable using a range of methods.

The erythrocytes may be successfully loaded with Mg2+ ions due to the formation of reversible pores and that they can be properly sealed by using a 0.9% NaCl solution. The Mg2+ was loaded into the erythrocytes in a study using the isotonic osmotic lysis method.[2] Another study was conducted with calcium-loaded erythrocytes to von

Willebrand factor by using erythrocytes from healthy, anonymized volunteers.[3] Amikacin was attempted to be loaded into human carrier erythrocytes as an antibiotic in one study.[4] Also, studies have shown that erythrocytes can be loaded with valsartan by utilizing the Preswell dilution procedure with sodium chloride as the medium and glutaraldehyde as the cross-linking agent.[5] An article in the Russian Open Medical Journal suggests the use of erythrocytes loaded with terpene-indole alkaloids as a medication carrier.[6] According to a study, erythrocytes loaded with alcohol oxidase can help in methanol detoxification.[7]

Patients received desferrioxamine as erythrocyte ghosts, and it was reported in human trials that there were no negative effects.[8] Furthermore, the literature suggests that immunophilin- loaded erythrocytes are a new delivery strategy for immunosuppressive drugs.[9] According to the study, isotonic resealing, which is based on hypotonic Preswell, has an encapsulation efficacy of 60%–80% when used to load primaquine phosphate into rat erythrocytes.[10]

Several methods have been used by researchers to load suitable candidates into erythrocytes. These methods include lipid fusion[11], method of endocytosis[12], method of Electro- insertion/electro encapsulation[13] and the methods of hypo osmotic lysis.[13–15]

Previous studies have used several immunoassay methods to analyze insulin concentration including chemiluminescence immunoassay (CLIA)[16], Enzyme-Linked Immunosorbent Assay (ELISA)[17], Radioimmunoassay (RIA)[18] and on-chip immunoassay.[19] Also, previous studies show using chromatographic assays for analyzing insulin concentration including high-performance liquid chromatography coupled with ultraviolet detection (HPLC- UV)[20], micellar electro kinetic capillary chromatography (MECC)[21], and liquid chromatography with tandem mass spectrometry (LC-MS/MS).[22] One study suggests a simple method for analyzing the insulin concentration of insulin present in camel milk by using UV-Vis Spectroscopy.[23]

The preliminary study introduces a novel concept: insulin-loaded erythrocytes as a biological dosage form. This endeavor has the potential to pave the way for future research, offering human erythrocytes as a promising answer to the critical issue of daily insulin administration for patients.

MATERIALS AND METHODS

ACTRAPID brand human insulin solution for injection (100 IU/mL) in 10 ml vials (Batch No. MT67D93, Novo Nordisk A/S, Novo Allé, DK-2880 Bagsvaerd, Denmark) and intravenous saline (NaCl 0.9% w/v) 500 mL containers (Batch No. A2309003, Axa Parenterals Ltd, India) were used in the study. Cary 60 UV-Visible spectrophotometer (Product No. G6860A, Agilent Technologies, made in Malaysia), trinocular microscopes (LABOMED, Labo America, USA), Magnus Micro Image Projection System (Magnus Analytics, New Delhi, India), a refrigerator (LG 242 L 3 Star Smart, South Korea), and a micro centrifuge (Sigma 1-14, Germany) were used at the Department of Pharmacy and Pharmaceutical Sciences, Faculty of Allied Health Sciences, University of Sri Jayewardenepura, Sri Lanka.

The ethical approval was obtained from the Ethics Review Committee of the Faculty of Medical Sciences, University of Sri Jayewardenepura, Gangodawila, Nugegoda, Sri Lanka, to draw blood from volunteers.

Develop a UV-Vis spectroscopy method to determine the concentration of soluble insulin.

A volume of 100 µL ACTRAPID human insulin solution for injection (100 IU/mL) was measured into a 5.00 mL volumetric flask by using a 10-100 µL pipette. It was diluted up to 5.00 ± 0.02 mL with 0.9% sodium chloride to yield 2 IU/mL solution. Then, it was stored at $2 – 8^0$ C in the refrigerator.

A UV-Vis cell was filled with the above standard insulin sample (2 IU/mL). Its spectrum was obtained in the wavelength range of 225 nm to 350 nm by using Cary Win UV Software for UV-Vis Applications. The wavelength of maximum absorption (λ_{max} =276 nm) was identified by using the software.

Standard dilution series of insulin were prepared as 2.0 IU/mL, 1.5 IU/mL, 1.0 IU/mL, and 0.5 IU/mL, and UV absorbance was measured at 276 nm (λ_{max}) in triplicate. The calibration curve of absorbance against different insulin concentrations was prepared, and the correlation of the calibration curve was obtained by using software called Statistical Package for the Social Sciences (SPSS version 16.0). A sodium chloride solution of 0.9% was used as a blank sample.

The data in the calibration curve was used to develop a simple application (the Insulin Concentration Calculator) by using the C computer programming language. When we give an absorbance value to the software, it will calculate the insulin concentration of an unknown sample according to the data from the standard samples. (Figure 1)

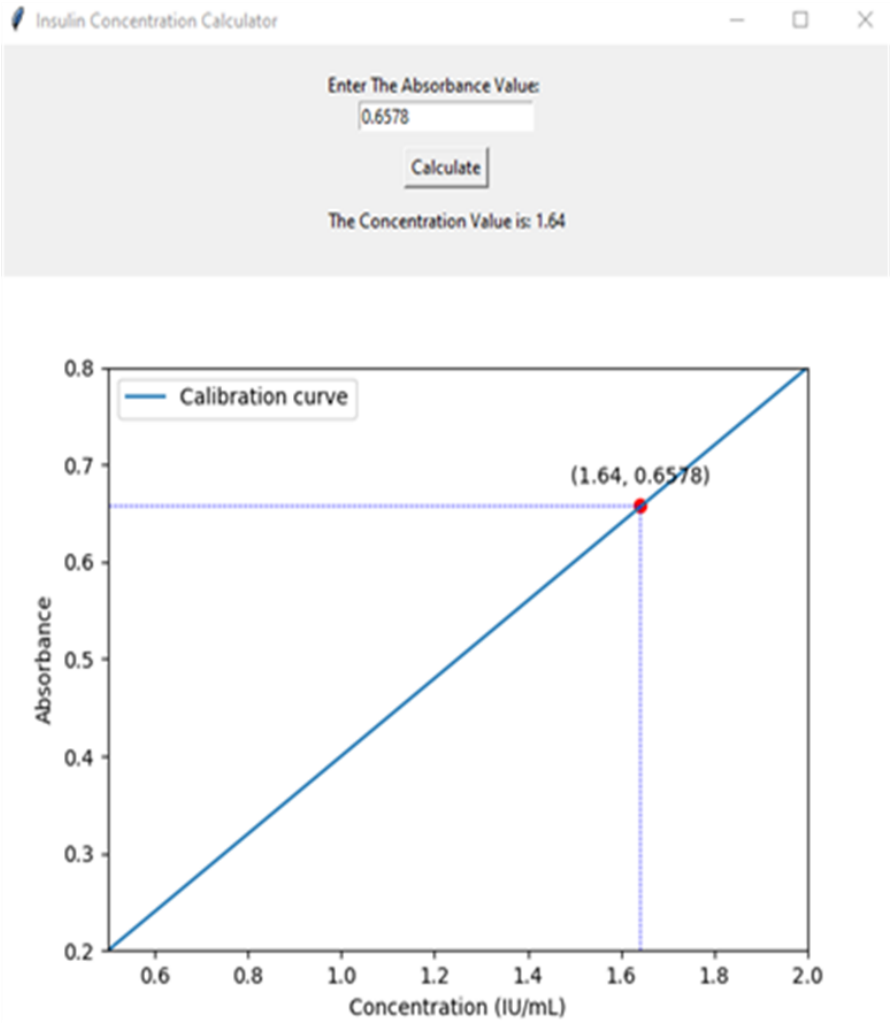

Figure 1: The result was given by the Insulin Concentration Calculator for an unknown diluted sample.

The sample with unknown concentration of insulin was diluted until it was in the range of 0.2–0.8 and the diluted factor was noted. Then the UV absorbance of the sample was observed using the UV-visible spectrophotometer at 276 nm. The value was entered into the insulin concentration calculator. The application gives the insulin concentration of the diluted, unknown sample. The concentration of the diluted sample was multiplied by the dilution factor to obtain the insulin concentration of the unknown sample.

Identifying optimum concentration of NaCl for making reversible pores for loading insulin into erythrocytes

After the volunteers contacted the investigator, the participant information sheets were given and explained the details of the research to the volunteers. A volume of 2.5 mL to 5 mL of fresh whole blood was drawn by venipuncture from the median cubital vein into EDTA tubes. It was then quickly stored at 4 °C for no longer than two days.

A volume of 1.5 mL of fresh blood was kept in a 1.5 mL centrifuge tube. Then, it was centrifuged for 5 minutes at 2500 RPM. After removing the supernatant, repeated the process twice by adding 0.75 mL of ice-cold 0.9% NaCl solution to wash the erythrocytes. A dilution series of NaCl was prepared as 0.8%, 0.7%, 0.6%, 0.5%, 0.4%, 0.3%, 0.2%, and 0.1% of NaCl by using intravenous NaCl solution 0.9% and distilled water. A total of 500.0 µL of the washed erythrocytes was mixed with 1.00 mL of a 0.9% NaCl solution. This process was repeated in triplicate with the remaining eight solutions (0.8%, 0.7%, 0.6%, 0.5%, 0.4%, 0.3%, 0.2%, and 0.1% of NaCl) and also distilled water. After 30 minutes, all ten solutions were centrifuged at 2500 RPM for 5 minutes.

The concentration of NaCl that started releasing hemoglobin was observed by naked eye and confirmed by obtaining the UV-Vis spectrum of hemoglobin in the range of 500 nm to 600 nm. (Figures 2 and 3) The optimum concentration of NaCl for making reversible pores on the erythrocyte's membrane (lysed erythrocytes) that can load insulin into the RBC with minimal leakage to the outside was identified.

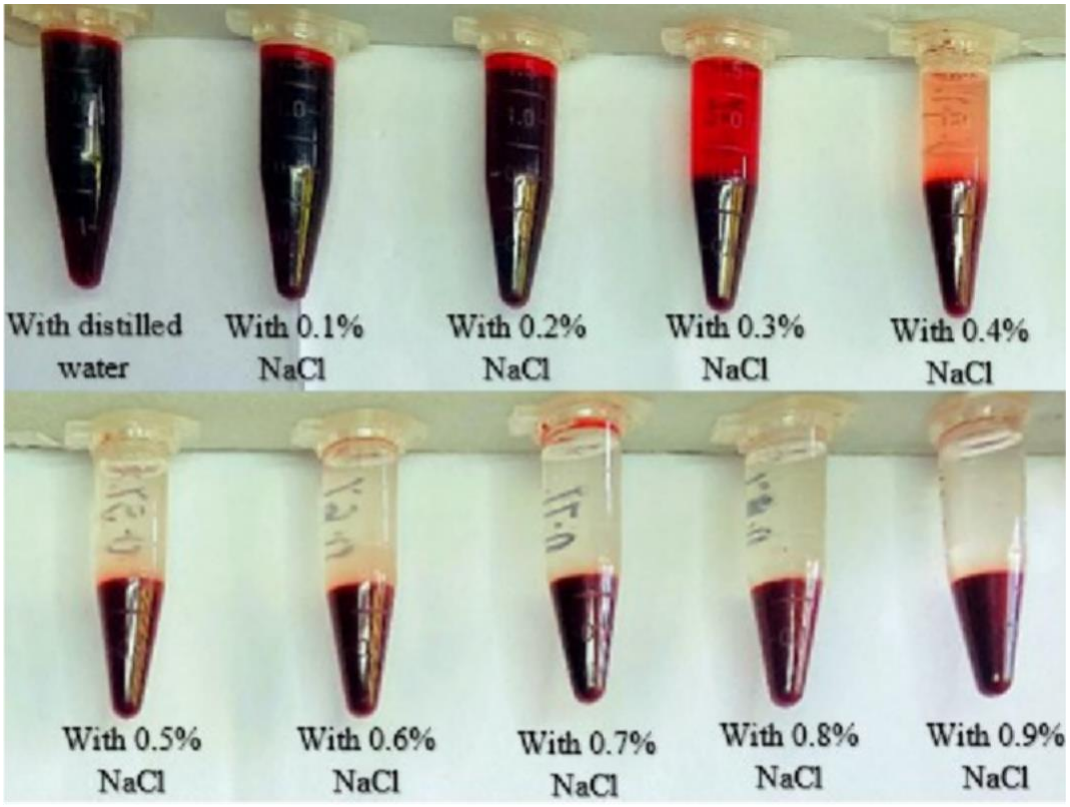

Figure 2: Erythrocytes after treated with distilled water, 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8% and 0.9% of NaCl.

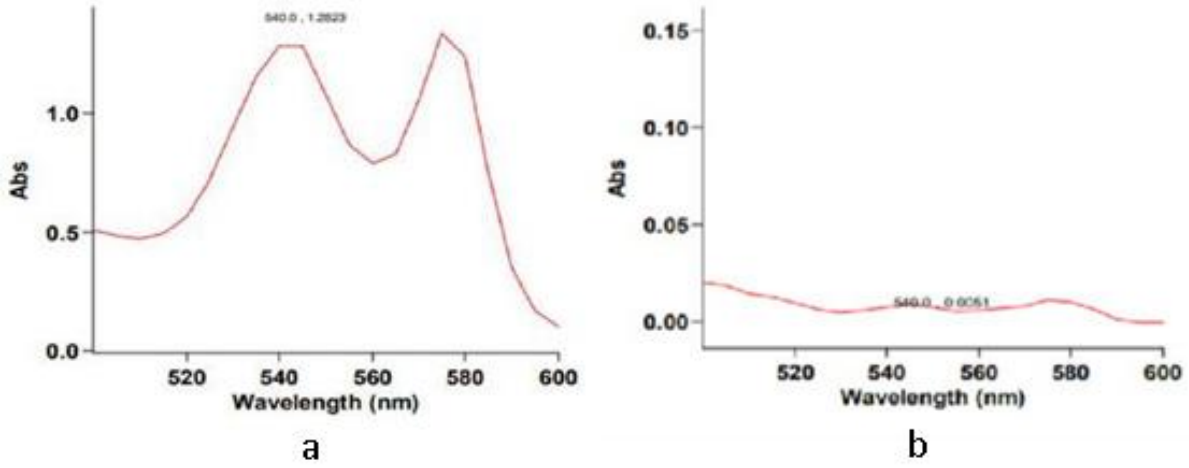

Figure 3: The spectrum of hemoglobin given by the supernatant of lysed erythrocytes in (a) 0.6% NaCl (b) 0.7% NaCl.

Identifying optimum insulin concentration of the medium to load insulin into the erythrocytes without damaging the cells

To identify the optimum insulin concentration to load insulin into the erythrocytes without damaging the cells, a dilution series of insulin was prepared in different concentrations: 80 IU/mL, 60 IU/mL, 40 IU/mL, and 20 IU/mL.

An amount of 500.0 µL of the washed erythrocytes were mixed with 1.00 mL of a 0.7% NaCl solution in a 1.5 mL micro-centrifuge tube. After 30 minutes, the suspension was centrifuged at 2500 RPM for 5 minutes. Lysed erythrocytes were withdrawn from the pellet. A volume of 300.0 µL of lysed erythrocytes was mixed with 300.0 µL of 80 IU/mL insulin in a 1.5 mL micro-centrifuge tube. Then, it was kept for 30 minutes with gentle shaking from time to time at 2–8 °C. Repeated the same procedure with 60 IU/mL, 40 IU/mL, and 20 IU/mL insulin solutions, all tests in triplicate. After 30 minutes, the four samples were centrifuged for 10 minutes at 1500 RPM. Then the optimum insulin concentration (20 IU/ml) was identified for the purpose of loading insulin into the erythrocytes without damaging the cells.

Determination of the amount of insulin that was loaded into human erythrocytes with time

A volume of 1.60 mL of lysed erythrocytes was prepared by using 0.7% NaCl. A volume of 0.80 mL of lysed erythrocytes was added into a 2.0 mL centrifuge tube. Then, 1.20 mL of 20 IU/mL soluble insulin was added to the centrifuge tube. After that, it was gently mixed from time to time and kept for 10 minutes to allow loading insulin into erythrocytes at 2–8 °C. Another two samples were similarly prepared, and kept for 20 and 30 minutes for loading insulin. Also, a blank sample was prepared with 1.20 mL of 0.7% NaCl instead of 20 IU/mL soluble insulin. After passing the relevant time, each test sample and its blank sample were centrifuged at 1500 RPM for 10 minutes. After centrifugation, 0.80 mL of supernatant was withdrawn from the test sample and the blank sample. Then, both were diluted with 0.9% NaCl up to 5.00 mL. Finally, the drop in insulin amount of the medium of the test sample was obtained by following the previously developed insulin concentration calculator and UV absorbance spectroscopy method. The same procedure was followed for the remaining samples that were kept for 20 minutes and 30 minutes and obtained their insulin concentrations. Those results were used to find the drop in the amount of insulin at 10 min, 20 min, and 30 min. The whole method was triplicated to ensure the repeatability of the results. (Table 1)

Table 1: The drop in amount of insulin in the medium and loaded amount of the insulin into erythrocytes with time.

Item	10 minutes diffusion			20 minutes diffusion			30 minutes diffusion		
Insulin in diluted supernatant (IU/mL) *	1.48	1.44	1.47	1.20	1.23	1.23	1.15	1.16	1.15
Insulin in supernatant (IU/mL)	9.25	9.00	9.19	7.50	7.69	7.69	7.19	7.25	7.19
Remain Insulin (IU) in 1.2 mL supernatant	11.10	10.8	11.03	9.00	9.23	9.23	8.63	8.70	8.63
Drop amount of Insulin (IU) from initial amount of 24 IU**	**13.02 ± 0.16**			**14.84 ± 0.13**			**15.35 ± 0.04**		
IU of insulin loaded into 0.6 mL of erythrocytes	4.10	4.30	4.15	4.20	4.45	4.45	4.40	4.50	4.95
IU of insulin / mL of isolated erythrocytes**	**6.97 ± 0.17**			**7.28 ± 0.24**			**7.69 ± 0.49**		

* *Dilution factor 6.25.*
** *Mean ± S.D.*

At the end of respective loading time periods, a volume of 0.60 mL of insulin-loaded erythrocytes was added into a 2 mL centrifuge tube. Then, 1 mL of 0.9% NaCl was added to each sample and kept 10 minutes at 2–8 °C for resealing the erythrocytes. After resealing the erythrocytes, those samples were centrifuged for 10 minutes at 1500 RPM to obtain resealed insulin-loaded erythrocytes. These were washed with 1.00 mL of a 0.9% NaCl solution and obtained as a pellet of insulin-loaded erythrocytes after centrifugation for 10 minutes at 1500 RPM.

To determine the amount of insulin loaded into erythrocytes, the palleted samples were mixed again with 1.00 mL of 0.7% NaCl and kept for 30 minutes to reopen the cells. After that, each sample was centrifuged for 10 minutes at 1500 RPM. Then 1.00 mL of supernatant was collected from each sample and pallet recovered.

Again, 1.00 mL of 0.7% NaCl was added, and after 10 minutes, each sample was centrifuged for 10 minutes at 1500 RPM. After that, another 1.00 mL of supernatant was collected from each sample. The process was repeated for each sample, and 3.00 mL of supernatant was collected. Then each sample was diluted to 5.00 mL with 0.9% NaCl, and the test samples were tested by following a previously developed insulin concentration calculator and UV absorbance spectroscopy method to find amount of insulin loaded into 0.6 ml of erythrocytes. Finally, the amount of insulin loaded into 0.6 ml of erythrocytes was divided by 0.6 ml to obtain IU of insulin/ml of isolated erythrocytes. The whole method was triplicated to ensure the repeatability of the results. (Table 1)

The one-way ANOVA test was done by using SPSS version 16.0 for the mean values of IU of insulin loaded into 1.00 mL of erythrocytes at 10 min, 20 min, and 30 min.

Stability of resealed soluble insulin-loaded erythrocytes in 0.9% NaCl medium at 2^0 C - 8^0 C with time

As control sample a total of 500 µL of the isolated washed unloaded erythrocytes were prepared by following the same method as above. The erythrocytes were combined with 1.00 mL of a 0.9% NaCl solution. The sample was stored at 2^0 C - 8^0 C in the refrigerator.

As test sample a total of 500 µL of resealed insulin-loaded erythrocytes were prepared by following the same method as above. The erythrocytes were combined with 1.00 mL of a 0.9% NaCl solution. The sample was stored at 2^0 C - 8^0 C in the refrigerator.

Then, the control and test samples were observed under the 400× magnification power of a trinocular microscope every 24 hours since the end of the loading process, and the stability was determined visually. Photographs were taken using a Magnus Micro Image System fixed to the light microscope. (Figure 4)

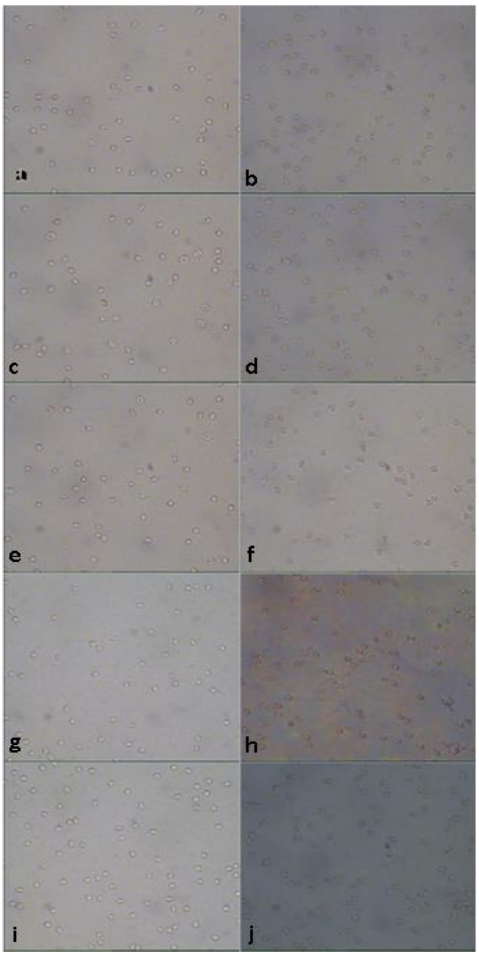

Figure 4: a, c, e, g and i are microscopic views of RBCs in the control sample after 24 hours, 48 hours, 72 hours, 96 hours and 120 hours. b, d, f, h and j are microscopic views of insulin-loaded RBCs in the test sample after 24 hours, 48 hours, 72 hours, 96 hours and 120 hours under 400× magnification.

RESULTS

Result of developing the UV-Vis spectroscopy method to determine the concentration of soluble insulin

The maximum UV absorption was 0.7490 at wavelength of 276 nm (λ_{max}) in the wavelength ranging from 225 nm to 350 nm. The UV absorbance of 2 IU/mL, 1.5 IU/mL, 1.0 IU/mL, and 0.5 IU/mL standard insulin samples were respectively 0.7442 ± 0.0204, 0.5521 ± 0.0164, 0.3815 ± 0.0104 and 0.1966 ± 0.0091. Each value represents the mean ± S.D. of triplicate.

The Pearson correlation of the calibration curve (the X axis is the insulin concentration in IU/mL, and the Y axis is the mean value of the absorbance in triplicate) was 0.998. The range of absorbance, 0.2 to 0.8, is well aligned with the Bear-Lambert law.

Results of identifying optimum concentration of NaCl for making reversible pores and optimum insulin concentration of the medium for loading insulin into erythrocytes

The supernatant of the erythrocytes treated with 0.6% NaCl gave the spectrum related to hemoglobin. (Figure 2, 3) The wavelength of maximum absorbance was 540 nm, and the absorbance value was 1.2823. The supernatant of the erythrocytes treated with 0.7% NaCl gave a spectrum as shown in figure 3. At the wavelength of maximum absorbance (540.0 nm), the absorbance value was 0.0051. A sodium chloride solution with a concentration of 0.7% was determined to be the optimum sodium chloride concentration to lyse erythrocytes and load insulin into erythrocytes without leaking hemoglobin.

Below is the decreasing percentage of leaking hemoglobin when increasing the concentration of NaCl from 0.6% to 0.7%. We assumed the UV-Vis absorbance at 540 nm was directly proportional to the quantity of hemoglobin and that all absorbance at 540 nm was absorbed only by hemoglobin. Absorbance at 540 nm was 1.2823 after treating with 0.6% NaCl solution. Absorbance at 540 nm was 0.0051 after treating with 0.7% NaCl solution.

The decreasing percentage of leaking hemoglobin= ((1.2823 - 0.0051)/ 1.2823)100 = 99.60%

The erythrocytes were fully disrupted after mixing with 80 IU/ml soluble insulin and 60 IU/ml soluble insulin. There were no separated pellets after centrifugation. Erythrocytes were partially disrupted with 40 IU/mL soluble insulin. Erythrocytes that were mixed with 20 IU/mL insulin solutions had less disruption to the cells. There was a clear, separated erythrocytes pellet after centrifugation. The 20 IU/mL insulin solution was identified as the optimum insulin concentration to transfer insulin into the erythrocytes without damaging the cells.

Calculating the drop in amount of insulin in the medium and loaded amount of the insulin into erythrocytes with time

There was no significant difference between the mean values of IU of insulin loaded into 1 mL of erythrocytes (6.97 ± 0.17, 7.28 ± 0.24, 7.69 ± 0.49) at 10 min, 20 min and 30 min ($P < 0.05$).

Results of stability of resealed soluble insulin-loaded erythrocytes in 0.9% NaCl medium at $2^0 – 8^0$ C with time

Even after 5 days, the cells in control sample remain fresh with normal contours. In the test samples, the insulin-loaded erythrocytes remained without bursting for 3 days (Figure 4). That is shown by the b, d, and f samples after 24 hours, 48 hours, and 72 hours. The samples h and j after 96 hours and 120 hours under 400× magnification showed the start of the busting of insulin-loaded erythrocytes. After 72 hours, the insulin-loaded RBCs started the degrading process. The stability period was set at 3 days' maximum in 0.9% NaCl medium at $2^0 – 8^0$ C.

DISCUSSIONS

ACTRAPID 10 mL was selected as the soluble insulin source. The product contains Zinc chloride, Glycerol, Metacresol, Sodium hydroxide, Hydrochloric acid and Water for Injections as excipients. The effects of the excipients were considered negligible for the whole study.

During the research, the new method to analyze insulin concentrations was used. There was little possibility of hemoglobin remaining in the supernatant due to the resealing technique. Here, the effect of hemoglobin was considered as negligible for the insulin assay method as their absorption maxima are widely different.

In this study, a minimum pore size that was able to transfer soluble insulin molecules inside the cells by simple diffusion was needed. The concentration of the hypotonic solution was gradually decreased to find the optimum concentration that could make the pore size large enough to transfer insulin.

UV absorbance was taken from the supernatant of erythrocytes after treatment with a 0.7% NaCl solution. It did not give the spectrum of hemoglobin. That confirmed there was no hemoglobin leaking after treatment with a 0.7% NaCl solution. So, we determined the pore size at a concentration of 0.7% NaCl was optimal to load insulin without leaking hemoglobin. (Figure 3)

When selecting, insulin suspensions were intentionally avoided because the solid particles present in a suspension could affect the insulin-loading process. Also, biphasic insulin was intentionally avoided due to separation difficulties.

The high concentration of soluble insulin and excipients can damage erythrocytes. However, a possible maximum insulin concentration was important because the loading process followed the diffusion principle. If we use a higher insulin concentration, the possible loading amount of insulin can be higher. To find that, we prepared a concentration gradient of soluble human insulin as 80 IU/mL, 60 IU/mL, 40 IU/mL, and 20 IU/mL.

CONCLUSION

In this study, it was possible to load 6.97 ± 0.17, 7.28 ± 0.24, and 7.69 ± 0.49 IU of insulin/mL of isolated erythrocytes at 10 min, 20 min, and 30 min of diffusion times with stability of three days at 2–8°C in 0.9% NaCl. According to the preliminary study results, there is a significant potential to use insulin-loaded erythrocytes as a novel insulin delivery system. Furthermore, in vitro and in vivo studies are essential before clinical use to identify the pharmacokinetics and pharmacodynamics of this biological dosage form. As well as improving storage conditions, formulation optimization should be conducted to increase the period of stability.

The UV absorbance-based application can analyze soluble insulin concentration. However, it is essential to conduct cross-validation against established methods, such as HPLC and ELISA, before this method can be used commercially.

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