Encapsulation of Porcine Insulin in Rabbit Erythrocytes and Its Disposition in the Circulation System in Normal and Diabetic Rabbits

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The application and usefulness of resealed erythrocytes as a cell carrier of porcine insulin were studied in rabbits. The insulin was loaded in rabbit erythrocytes by a dialysis method followed by isotonic resealing of the erythrocytes at 25 and 37 °C. The amounts of insulin entrapped in the erythrocytes at these temperatures were 6.23 ± 1.05 and 5.89 ± 1.33 IU/ml of packed cell, with efficiency of encapsulation being 6.0 ± 1.2 and 5.8 ± 2.0%, respectively. When the insulin loaded erythrocytes were incubated in phosphate buffered saline at 37 °C, the insulin levels in the resealed cells declined according to a biexponential function. The in vitro stability of the cells resealed at 25 °C was superior to that at 37 °C. After subcutaneous (s.c.) administration of free insulin, the absorption rate constants in diabetic rabbits were larger than those of normal rabbits. The in vivo decline of insulin released from the loaded erythrocytes indicated a biexponential kinetics. Although the pharmacokinetic behavior of plasma insulin between normal and diabetic rabbits was similar, the hypoglycemic effect was very different between them. These insulin dosings, be they intravenous (i.v.) dosing of insulin or loaded cells or s.c. dosing of insulin, maintained the hypoglycemic effect in diabetic rabbits about twice as long as in a normal animal. Moreover, the effective period of insulin-loaded erythrocytes was longer than that of free insulin dosings. These results suggest that the insulin-loaded erythrocytes may be useful as a dosage form for treatment of patients with diabetes mellitus.

Keywords — insulin loaded erythrocyte; alloxan diabetes; hypoglycemic effect; blood glucose; rabbit erythrocyte; enzyme immunoassay; pharmacokinetics

Introduction

The systemic administration of free drugs to patients under certain circumstances suffers serious disadvantages. The drug may undergo premature degradation, inactivation or excretion with the consequent loss of pharmacological activity. Alternatively, it may be necessary to protect the host from unwanted immunological or pharmacological effects. Endogenous peptides with specific biological effects have attracted attention as new drugs. Insulin, administered subcutaneously, has been used as the primary treatment for severe diabetes for several years, even though effective methods have been described for oral,¹ nasal,² aerosol,³ and rectal administration.⁴

Attempts have been made to overcome the problems mentioned by encapsulating drugs in microcapsules for use in vivo. Both synthetic and biosynthetic materials have been used in the preparation of microcapsules. Synthetic materials used include polyamide, polyurea, polyurethane, epoxy resin, ethyl cellulose, polystyrene, silicon rubber and gelatin.⁵,⁶ Problems encountered with the use of these materials include the inability of the body to biodegrade many of those used and a failure to produce capsules small enough not to obstruct the microcirculation. Biodegradable materials include the well-known liposomes which consist of a synthetic membrane modelled after natural membranes⁷ and a polylactic acid which is biodegradable.⁸ Attempts have been made to use the body’s own cells as drug carriers. Erythrocytes are versatile carriers capable of entrapping a variety of drugs ranging from small molecules to polypeptides.⁹–¹₀

In this paper we have described a method whereby rabbit erythrocytes may be loaded with porcine insulin. The pharmacological activities of the preparation in rabbits have been investigated together with pharmacokinetic behavior of the encapsulating erythrocytes.

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Materials and Methods

Materials — Male Japanese white rabbits weighing 3.8—4.2 kg were used throughout this experiment. Porcine insulin (27.1 IU/mg) was the generous gift of Novo Pharmaceutical Co. (Tokyo, Japan). Peroxidase (POD, type VI, about 300 IU/mg) and bovine serum albumin (fraction V, RIA grade) were purchased from Sigma Chemical Co. (Mo., U.S.A.). Alloxan (monohydrate) was purchased from Nacalai Tesque (Kyoto, Japan). All other chemicals used in this study were of reagent grade.

Determination of Insulin by Enzyme Immunoassay — The anti-insulin antibody was prepared by the inoculation of porcine insulin with Freund's complete adjuvant to guinea pigs according to the method of Hashimoto and Kawano. The antibody was conjugated with POD by the method of Nakane and Kawaoi. The measurement of insulin concentrations in plasma and erythrocytes was carried out by the method of Iwasaki et al. using a microplate with 96 wells for enzyme immunoassay (Corning Co., U.S.A.). Four-tenths of a ml of anti-porcine insulin antibody solution (0.5 mg/ml of 10 mM sodium phosphate buffer, pH 7.4) was added into each well of the plate and stored overnight at 4°C. The antibody solution was discarded and the plate was washed 3 times with 0.4 ml of buffer W (50 mM Tris-HCl, pH 7.6, containing 0.15 M NaCl and 0.05% Tween 20). Fifty μl of sample solution (plasma, diluted hemolysate, or standard insulin) and 250 μl of buffer A (10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl, 0.1% bovine serum albumin and 0.01% sodium azide) were added into each well of the plate. The plate was incubated for 2 h at 37°C in a thermostatically controlled box and washed 3 times with 0.4 ml of buffer W. The POD-labeled antibody solution (300 μl), diluted 10000-fold with buffer A, was added into each well and incubated for 2 h at 37°C. The plate was washed 3 times with 0.4 ml of buffer W and POD activities on the plate were assayed by the method of Ferrua et al. The insulin contents in samples were calculated by using the insulin standard curve simultaneously obtained.

Determination of Plasma Glucose — Plasma glucose levels were measured by the method using the glucose oxidase–peroxidase reaction.

Encapsulation of Insulin in Erythrocytes — Packed rabbit erythrocytes (about 10 ml), which had been washed three times in phosphate buffered saline and centrifuged, were dialyzed against 3 l of 10 mM sodium phosphate buffer (pH 7.0) for 2 h at 4°C. The insulin solution, in which 20 mg of porcine insulin was dissolved in 1.2 ml of 0.01 N HCl, followed by adding 1.8 ml of 0.05 M sodium phosphate buffer (pH 7.4), was mixed with 5 ml of erythrocyte ghost suspension and agitated gently for 2 h at 4°C. The isotonicity was restored by adding a calculated amount of 0.1 M sodium phosphate buffer (pH 7.4) containing 1.54 M NaCl and 50 mM glucose. This suspension was incubated for 30 min at 25 or 37°C. After washing 4 times with 5 ml of isotonic saline solution, the loaded erythrocytes were suspended in saline (3.0 IU/ml) and injected into the experimental animals.

Induction of Alloxan Diabetic Rabbit — Four male rabbits weighing 3.5—4.0 kg were injected intravenously with alloxan monohydrate (120 mg/kg) 2 weeks before the study to induce experimental diabetes. Diabetes mellitus was confirmed in each rabbit by documenting persistent hyperglycemia (mean plasma glucose, 450 mg/dl) and glycosuria.

In Vitro Stability of Insulin-Loaded Erythrocytes — The cells packed with insulin were diluted 20-fold with rabbit erythrocytes resesed without insulin. A half ml of cell suspension (about 20% of hematocrit value) was added into an equal volume of phosphate buffered saline and incubated at 37°C. After the incubation the suspension was cooled in an ice bath for 10 min and centrifuged for 800 × g. The cells were washed twice with 2 ml of cold isotonic saline and lysed by mixing with 0.9 ml of cold distilled water. The hemolysate was sonicated for 2 min with a Bransonic 12 sonicator (Branson Cleaning Equipment Co., U.S.A.). This solution was diluted 2000-fold with the immunoassay medium (buffer A). The insulin concentration in erythrocytes was determined by the enzyme immunoassay described above.

Administration Routes of Insulin — The
free insulin and loaded erythrocytes were injected into a marginal vein of the rabbit ear, and free insulin was also injected subcutaneously on the animal's rump.

**Statistical and Data Analyses** — The data obtained following a single i.v. dose of free insulin and insulin-loaded erythrocytes were analyzed using one- and two-compartment open models that are described by the following respective equations:

\[ C = C_0 \cdot e^{-k_e t} \]  \hspace{1cm} (1)

\[ C = A \cdot e^{-\alpha t} + B \cdot e^{-\beta t} \]  \hspace{1cm} (2)

where \( C \) and \( C_0 \) are the insulin concentration in plasma at time \( t \) and 0, respectively, \( k_e \) is elimination rate constant, \( \alpha \) and \( \beta \) are rate constants during the initial rapid and terminal slower phases of the curve, respectively, with \( A \) and \( B \) being the respective zero-time intercepts.

The data obtained following a single s.c. dose of insulin were analyzed using a one-compartment open model with first-order absorption process that is described by the following equation:

\[ C = \frac{f \cdot D \cdot K_a}{V_d \cdot (k_a - k_e)} \cdot (e^{-k_et} - e^{-k_et}) \]  \hspace{1cm} (3)

where \( f \) is fraction of absorption, \( D \) is dose, \( V_d \) is distribution volume, and \( k_a \) is absorption rate constant. Here parameters, \( f \) and \( k_a \) were estimated by fixing them at the values of \( V_d \) and \( k_e \) obtained from i.v. experiment. Results are expressed as mean \( \pm \) S.D. Statistical analysis was performed by the non-paired Student's \( t \)-test and \( p \)-value of 0.05 or less was considered significant. Regression lines were calculated using an iterative non-linear least squares regression analysis (MULTI).\(^{16}\)

**Results and Discussion**

**Recovery of Insulin in the loaded Erythrocytes**

In a previous study we used modified Hank's solution for the resealing of erythrocytes.\(^9,^{10}\) In this study, CaCl\(_2\) was omitted from the resealing solution (hypotonic phosphate buffer) due to problems with precipitation. However, the omission had no effect on the resealing process and subsequent stability of cells.

On encapsulation of porcine insulin into rabbit erythrocytes, the optimum ratio of insulin to the packed cell volume (20 mg of insulin/5 ml of packed cells) was observed. The encapsulation was performed at two different temperatures, 25 and 37 °C. Under the optimum condition the recovery of insulin in the loaded erythrocytes resealed at 25 °C was 6.0 ± 1.2%; insulin content in the erythrocytes was 6.23 ± 1.05 IU/ml of packed cells. Similar recovery (5.8 ± 2.0%) and content of insulin (5.89 ± 1.33 IU/ml of packed cells) were obtained at 37 °C. A low insulin content in the loaded erythrocytes (about 6% of applied amount) and optimum insulin/cell volume ratio for the encapsulation were observed in this experiment. This result suggests that insulin may bind to the membrane fragments or hemoglobin and partly prevent the resealing process. In consideration of erythrocyte membrane consisting of a lipid bilayer as well as unilamellar liposomes, similar results have been reported about insulin-encapsulated liposomes in which insulin nonspecifically binds to the surface of a liposome and insulin-mediated aggregation and/or fusion of liposomes subsequently occur.\(^{17}\)

**In Vitro Stability of Insulin-Loaded Erythrocytes**

The stability of insulin-loaded erythrocytes

![Fig. 1. In Vitro Stabilities of Insulin-Loaded Erythrocytes Resealed at Different Temperatures](image-url)

○, resealed at 25 °C; ●, resealed at 37 °C.
### Table 1. Parameters for Stability of Insulin-Loaded Erythrocytes Resealed at 25 and 37 °C

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Resealed at 25 °C</th>
<th>Resealed at 37 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A$ (mIU/ml)</td>
<td>315.1 ± 26.0</td>
<td>226.7 ± 14.5</td>
</tr>
<tr>
<td>$k_{d1}$ (min$^{-1} \cdot 10^{-9}$)</td>
<td>56.8 ± 4.7</td>
<td>128.5 ± 27.9$^a$</td>
</tr>
<tr>
<td>$t_{1/2}$ (min)</td>
<td>12.16 ± 1.07</td>
<td>5.41 ± 1.18$^a$</td>
</tr>
<tr>
<td>$B$ (mIU/ml)</td>
<td>18.8 ± 3.0</td>
<td>61.0 ± 15.3</td>
</tr>
<tr>
<td>$k_{d2}$ (min$^{-1} \cdot 10^{-9}$)</td>
<td>1.42 ± 0.93</td>
<td>14.15 ± 4.37$^b$</td>
</tr>
<tr>
<td>$t_{2/1/2}$ (min)</td>
<td>495.0 ± 104.6</td>
<td>49.15 ± 15.34$^b$</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.D. of 3 experiments. Significantly different from the corresponding value at 25 °C, $a)$ $p<0.01$ and $b)$ $p<0.05$.

![Graph A and B](image_url)

**Fig. 2.** Time Courses of Plasma Insulin Concentrations after i.v. and s.c. Administrations of Free Insulin into Normal and Diabetic Rabbits

A) Normal rabbit (●, i.v. dosing of 0.15 IU/kg; ○, s.c. dosing of 1.00 IU/kg). B) Diabetic rabbits (●, i.v. dosing of 0.30 IU/kg; ○, s.c. dosing of 2.00 IU/kg).

### Table II. Pharmacokinetic Parameters of Insulin in Plasma after i.v. and s.c. Administrations of Free Insulin

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal$^a$</th>
<th>i.v.</th>
<th>s.c.</th>
<th>Diabetes$^b$</th>
<th>i.v.</th>
<th>s.c.</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_0$ (μIU/ml)</td>
<td>566.3 ± 28.2</td>
<td>764.5 ± 56.4</td>
<td></td>
<td>0.090 ± 0.007</td>
<td>0.093 ± 0.007</td>
<td></td>
</tr>
<tr>
<td>$k_c$ (min$^{-1}$)</td>
<td>7.70 ± 0.58</td>
<td>7.45 ± 0.56</td>
<td></td>
<td>264.9 ± 13.2</td>
<td>353.2 ± 26.1$^c$</td>
<td></td>
</tr>
<tr>
<td>$t_{1/2}$ (min)</td>
<td>9.69 ± 2.07</td>
<td>15.38 ± 1.77$^c$</td>
<td></td>
<td>6.46 ± 0.70</td>
<td>18.34 ± 0.83</td>
<td></td>
</tr>
<tr>
<td>$V_k$ (ml/kg)</td>
<td>8.74 ± 0.16</td>
<td>23.27 ± 1.02</td>
<td></td>
<td>6.46 ± 0.16</td>
<td>18.34 ± 0.83</td>
<td></td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.D. of 4 experiments. $a)$ i.v. dose: 0.15 IU/kg; s.c. dose: 1.00 IU/kg. $b)$ i.v. dose: 0.27 IU/kg; s.c. dose: 1.79 IU/kg. $c)$ Significantly different from the corresponding value of normal rabbit at $p<0.01$. 
Insulin Encapsulated in Erythrocytes

Fig. 3. Time Courses of Plasma Insulin Concentrations after Administrations of Insulin-Loaded Erythrocytes into Normal and Diabetic Rabbits
A) Normal rabbit (3.0 IU/kg). B) Diabetic rabbit (6.0 IU/kg). Each value represents the mean of 4 experiments. Bars show S.D. of each point.

was investigated by their suspension in phosphate buffered saline (Fig. 1 and Table I). The insulin levels in the erythrocytes resealed at both 25 and 37 °C decreased according to a biexponential function. Although the $k_{e1}$ (initial elimination rate constant) of the erythrocytes resealed at 25 °C was similar to that at 37 °C, its $k_{e2}$ (terminal rate constant) at 25 °C was lower. This result indicates that the loaded erythrocytes resealed at lower temperature are more stable than those at high temperature at a later period. We tried to determine insulin levels in buffer medium released from the erythrocytes, but these could not be determined because of the interference of hemoglobin. The reason in the experiment on in vitro stability that the insulin levels in buffer medium could not be determined is that hemoglobin released from the erythrocytes probably interfered with the immunoassay system used. A similar finding was shown in a previous paper. 10 For the in vitro stability experiment, therefore the insulin concentrations in erythrocytes were determined using diluted hemolysate (2000-fold dilution). On the other hand after i.v. administration of insulin-loaded erythrocytes the levels in plasma could be determined, but not in erythrocytes. This may be due to the loaded erythrocytes probably being diluted with many other intact cells in the systemic circulation, after which hemoglobin released from the loaded cells is trapped by the reticulo-endothelial system.

Elimination of Insulin after i.v. Administration of Insulin-Loaded Erythrocytes and Free

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal $\alpha$</th>
<th>Diabetes $\beta$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (μIU/ml)</td>
<td>820±86</td>
<td>1095±120</td>
</tr>
<tr>
<td>α (min$^{-1}$)</td>
<td>0.130±0.028</td>
<td>0.055±0.012 $c$</td>
</tr>
<tr>
<td>$t_{α/2}$ (min)</td>
<td>5.33±1.15</td>
<td>12.60±2.75 $c$</td>
</tr>
<tr>
<td>B (μIU/ml)</td>
<td>209.9±35.6</td>
<td>148.8±30.3</td>
</tr>
<tr>
<td>β (min$^{-1}$·10$^{-9}$)</td>
<td>2.93±1.67</td>
<td>1.62±0.69</td>
</tr>
<tr>
<td>$t_{β/2}$ (h)</td>
<td>3.94±2.15</td>
<td>7.12±3.04</td>
</tr>
<tr>
<td>AUC (mIU/ml·min)</td>
<td>80.45±5.67</td>
<td>119.84±11.26</td>
</tr>
<tr>
<td>Bioavailability (%)</td>
<td>62.26±4.39</td>
<td>61.70±5.80</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.D. of 4 experiments. $a$) Dose: 3.0 IU/kg. $b$) Dose: 6.0 IU/kg. $c$) Significantly different from the corresponding value of normal rabbit at $p<0.01$. 

TABLE III. Pharmacokinetic Parameters of Insulin in Plasma after i.v. Administration of Insulin-Loaded Erythrocytes
Insulin and after s.c. Administration of Free Insulin in Normal and Diabetic Rabbits

The plasma decay curve of insulin and pharmacokinetic parameters after i.v. and s.c. administrations of free insulin are shown in Fig. 2 and Table II, respectively. After the i.v. administration into both normal and diabetic rabbits, the plasma concentrations decreased according to a monoexponential function. No significant differences in $k_e$ and $t_{1/2}$ were observed between normal and diabetic animals; similar results have been reported in human.\(^{18}\) However, after s.c. administration of free insulin the $k_a$ value in diabetic rabbits was 1.5 times higher than that in normal animals ($p < 0.01$). It is suggested that in subcutaneous tissues of diabetic rabbits the degradation of insulin may be less and/or the absorption through their blood vessels may increase. Therefore, the use of subcutaneous injection of insulin for clinical usage has been difficult for assessment of the kinetics of its absorption in patients with various stages of diabetes.\(^{19}\)

### Table IV. Changes of Plasma Glucose Levels and Duration of Hypoglycemic Effect after i.v. and s.c. Administration of Free Insulin and i.v. Administration of Insulin-Loaded Erythrocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Difference of glucose concentration (^{a)}) (mg/dl)</th>
<th>$T_{\text{min}}$ (^{b)}) (h)</th>
<th>Duration time (^{c)}) (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Normal rabbit</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free insulin (i.v.)</td>
<td>95±12</td>
<td>0.34±0.05</td>
<td>—</td>
</tr>
<tr>
<td>Free insulin (s.c.)</td>
<td>53±11</td>
<td>1.22±0.31</td>
<td>—</td>
</tr>
<tr>
<td>ILE (^d)} (i.v.)</td>
<td>98±18</td>
<td>0.52±0.11</td>
<td>—</td>
</tr>
<tr>
<td><strong>Diabetic rabbit</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free insulin (i.v.)</td>
<td>304±26</td>
<td>1.14±0.27</td>
<td>1.3±0.2</td>
</tr>
<tr>
<td>Free insulin (s.c.)</td>
<td>488±68</td>
<td>3.19±0.43</td>
<td>3.7±0.8</td>
</tr>
<tr>
<td>ILE (^d)} (i.v.)</td>
<td>532±57</td>
<td>2.03±0.19</td>
<td>4.0±0.7</td>
</tr>
</tbody>
</table>

Doses are the same as those described in Tables II and III. Each value represents the mean ± S.D. of 3 experiments.

\(^a)}\) Differences between the concentration before dosing insulin and the minimum glucose concentration after treatment.

\(^b)}\) Time required for plasma glucose concentration to reach minimum level.

\(^c)}\) Time during which the plasma glucose level is less than 250 mg/dl.

\(^d)}\) Insulin-loaded erythrocytes.
After i.v. administration of insulin loaded erythrocytes, the plasma concentrations in normal and diabetic animals decreased according to a biexponential function including two decline rate constants which are related to the degradation of unstable cells and slow release of insulin from relatively stable cells (Fig. 3). The two elimination rate constants, $\alpha$ and $\beta$, in diabetic animals were smaller than those in normal animals, though significant difference in $\beta$ value was not observed between the two groups (Table III). Moreover, the half life of the loaded cells obtained from the in vitro stability experiment was similar to that after i.v. dosing of the loaded cells into diabetic rabbits, but the values were larger than those of the loaded cells in normal animals. These results suggested that the long half life of insulin after i.v. dosing of loaded cells into diabetic rabbits may be due to the lowered function of the reticulo-endothelial system.

**Changes of Plasma Glucose Concentration after Administration of Insulin**

Figure 4 shows the pharmacological activity of insulin determined by measuring the plasma glucose concentrations after administration of various insulin preparations, and Table IV summarizes various parameters obtained. In normal rabbits, though the amount of insulin in the loaded cells administered was 20 times higher than that of free insulin, no significant difference was observed in the profiles of plasma glucose levels between normal and diabetic rabbits. In diabetic rabbits, after i.v. (free insulin and loaded cells) and s.c. (free insulin) administrations, a delayed initiation of the effect and elongation of the effective time were observed in comparison with those in normal animals. Initiation of the hypoglycemic effect was observed in the early stage following i.v. administration of the insulin-loaded erythrocytes. This suggests that the early initiation may be the result of the degradation of unstable cells and that the subsequent duration of the effect may be due to degradation of relatively stable cells and prolonged release of insulin from these cells. Although the administration route of insulin is generally subcutaneous, the initiation of hypoglycemic effect is slow (about 1–2 h after s.c. injection). In this study the initiation of the effect after i.v. administration of the loaded erythrocytes was as early as that after i.v. dosing of free insulin, and the duration was longer than that after s.c. administration of free insulin. The differences between plasma glucose level before dosing and minimum level after dosing were largest after i.v. dosing (6.0 IU/kg dose) of the loaded erythrocytes in diabetic rabbits. Retention of the hypoglycemic effect below 250 mg of glucose/dl was longest after administration of the loaded erythrocytes by this means among the three way tested. These results suggest that the insulin-loaded erythrocytes as a carrier may be useful for the treatment of diabetes, although some problems such as manner of preparation of encapsulated cells from which insulin is gradually released remain to be solved.

For the ease of experiments we chose rabbit as a experimental animal in this study. Considering the high recovery of insulin and stability of the loaded erythrocytes, human erythrocytes or other animal cells such as those from dog, mouse and pig should next be used. Our next investigations will be on the encapsulation of insulin into human erythrocytes and the long-term freezing storage using a high concentration of glycerol.

**References**


