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A new drug form of blood coagulation factor IX: Red blood cell-entrapped factor IX

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D Data Interpretation
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PI

Summary

Background: Deficiency of factor IX causes hemophilia B, and primary treatment for hemophilia B is based on recurrent infusions of deficient factor IX. Frequent infusions of foreign protein diminish patients' quality of life, and increase the risk of development of immune reaction. We entrapped factor IX into erythrocytes-carriers (pharmacocytes) to prolong the drug's circulation life time, and to prevent immune response to the drug.

Material/Methods: Factor IX was biotynilated by standard method and then loaded aseptically into volunteers' erythrocytes with our gentle procedure of stepwise dialysis. The comparison of pharmacokinetics for free and autologous erythrocytes-entrapped biotinylated factor IX (FIX_{biot}) was done. Concentrations of factor IX_{biot} in plasma and lysates of erythrocytes were quantitatively assessed with a sandwich ELISA.

Results: Stepwise dialysis method allowed stable loading of factor IX_{biot} into erythrocytes. Elimination of the loaded erythrocytes followed the first-order kinetics. The mean half-time of elimination for free FIX_{biot} was 8.8±5.6 hours, and for RBC-entrapped factor IX_{biot} 73.9±16.0 hours. Elimination of FIX_{biot} from plasma did not follow the first order kinetics because this factor concentration depended not only on the rate of its elimination, but also on the rate of factor appearance in plasma as a result of pharmackocytes' degradation. A rough estimate of the feasibility of the approach was done.

Conclusions: The life time of the erythrocyte-based form of FIX_{biot} in the circulation is significantly (5–10 times) prolonged compared with its free form, suggesting that this form has potential clinical applications.

key words: drug delivery • erythrocytes-carriers • coagulation factor IX • stepwise dialysis method • pharmacokinetics

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BACKGROUND

Hemophilia is one of the most common disorders of the blood coagulation system, caused by missing or insufficient activity of one of the coagulation factors. The 2 most common types of hemophilia are hemophilia A and hemophilia B, which result from deficiencies in factor VIII (FVIII) and factor IX (FIX) activity, respectively. Basic treatment for hemophilia is based on recurrent infusions of the missing coagulation factor. If hemophilia is severe (less than 1% of the normal factor activity), 2 or 3 infusions per week may be required. Frequent infusions diminish patients' quality of life and increase the risk of treatment-associated complications because blood-derived products are not absolutely safe in terms of blood-borne virus transmission, and because frequent infusions of foreign proteins may provoke an immune response in patients. An inhibitory antibody can arise against the factor injected. This occurs more frequently in hemophilia A patients (20–33% of cases of moderate and severe hemophilia), than in hemophilia B (1–6% of cases). Appearance of inhibitors in hemophilia B is often related to allergic reactions to factor IX infusions [1,2].

Modern pharmacology continually develops new approaches to prolong a drug's circulation life time, to provide the drug target delivery, reduce toxicity, and/or to prevent immune response to the drug. Various approaches may be tried toward improving currently used antihemophilic blood products. At present, recombinant FVIII and FIX are being developed, with which there is no risk of transmitting viruses [3,4]. Attempts are being made to produce antihemophilic factors with a mutation that makes their molecules more resistant to natural degradation in the circulation [5]. As well, work is being done on gene therapy for hemophilia [6]. There are alternative approaches for treating bleeding in patients with inhibitory hemophilias: activated prothrombin complex concentrate (FEIBA or Autoplex) at 50–100 U/kg / each 6–24 h (but no more than 200 U/kg/24 h) [7], antifibrinolytics (ε-aminocaproic acid or tranexamic acid) [8], and immunomodulators (prednisolone, Cytoxan, IVIg, Rituximab, etc.) [9,10]. Sometimes plasmapheresis is used to remove inhibitory antibodies, which causes immediate, but short-lived effects [11–13]. Currently, inhibitory hemophilia is successfully treated by recombinant activated factor VII (rFVIIa) (NovoSeven) (90–120 μg/kg each 2–6 hours until hemostatic control is established) [14–16]. Its mechanism of action is presently being discussed [17–21]. Factors VIIa and VII have similar affinity to tissue factor, but only complex of tissue factor with factor VIIa, extrinsic tenase, is the physiological activator of coagulation. Some researchers suggest that coagulation improves after infusion of high-dose activated factor VIIa because it out-competes factor VII. It results in increase of extrinsic tenase concentration, and leads to significant acceleration of coagulation [17,18]. There is, however, an alternative view on the procoagulant action of rVIIa [19–21], based on the observation that high doses of fVIIa can directly (without tissue factor) activate factor X on the surface of activated platelets [19,20]. RVIIa can be used to stop urgent bleeding, but its time of action is short ($t_{1/2}$ in plasma is ~2.5 hours), and its price is high.

In this study, we suggest a basically different approach based on the use of autologous red blood cells (RBC) as carriers for factor IX.

Autologous erythrocytes are very suitable drug carriers. They are biocompatible and biodegradable cells, which have long life time in circulation, and can be easily isolated in large quantities. The advantages of these cells as drug carriers are [22–24]:

1. The drug inside the cell is prevented from inactivation in plasma.
2. The duration of drug circulation is significantly increased. Theoretically, it can reach that of a normal erythrocyte, i.e., 90–100 days; and during this period an almost constant concentration of the drug in blood is maintained.
3. Immune system response against a drug inside the erythrocyte-carrier is greatly reduced.

Properties of erythrocyte carriers depend on their preparation. There are many techniques to load these cells, and we can choose a technique suited to the aim [25], mainly using various hypoosmotic approaches (reversible hypoosmotic lysis, dialysis, preswelling method, stepwise dialysis [24–30]). They all use decrease of a medium tonicity to create pores in the erythrocyte membranes for admission of a drug into the cells. Subsequently the cells are "sealed" by increasing tonicity up to the normal level.

There are methods of drug inclusion that use high-voltage dielectric breakdown of erythrocyte membrane leading to pore formation [31], as well as simple incubation of erythrocytes in a medium containing the drug [32,33]. The first method is not widely used due to labor-intensiveness and impossibility of obtaining large quantities of loaded erythrocytes. The second method is used to load erythrocytes with some anti-tumor antibiotics, but it is not suitable for loading membrane-impermeable drugs such as proteins.

There are reports of loading erythrocytes with enzymes that should work with substrate that is present in blood plasma and that easily crosses the cell membranes (the best-known example is asparagin – the substrate of asparaginase) [28]. In these cases, the loading technique should be as gentle as possible to provide cells retaining long-term circulation viability [29,30].

If the loaded drug is to act in plasma, then maintaining its concentration at the therapeutic level is possible either via diffusion from the cell or steady disruption of the erythrocytes in blood [34].

As the majority of erythrocytes are removed from the circulation by either the spleen or liver, the fate of the loaded drug is not a priori clear. In our study, we, for the first time, tried to determine whether erythrocytes may be carriers for enzymes that have to work in the circulation outside the carrier cells, i.e., whether these carriers can maintain a significant level of the loaded drug in plasma. The antihemophilic factor IX is an example of this kind of enzyme. Although hemophilia B is a rarer disease than hemophilia A, we chose factor IX for loading of erythrocytes because its life time is longer than that of factor VIII ($t_{1/2}$ is 24 and 12 hours, respectively [35]). This factor also has smaller molecular weight, allowing for a more efficient inclusion. In addition, factor IX is known to cause allergic reactions [1,2], but the risk of immune response for preparation into the erythrocytes-carriers is likely to be much reduced - most of the foreign protein is concealed within self cells and thereby escapes immune recognition.

Loading of factor IX into erythrocytes is likely to allow longer intervals between infusions, because the protein is injected within vehicle cells (pharmacocytes), rather than in a free form. A portion of the pharmacocytes in the circulation are destroyed naturally at a low rate, releasing the factor into the blood. The factor concentration within the cells can be manipulated so as to maintain the needed therapeutic level of the released factor in the blood (5–10% of the norm) [36].

In order to monitor infused factor IX, it was biotinylated. This study describes the procedure for inclusion of biotinylated factor IX (FIX_{biot}) into normal donor or autologous red blood cells and compares the pharmacokinetics ($t_{1/2}$) of free FIX_{biot} and FIX_{biot} entrapped in red blood cells. The results suggest that the circulation life time of the pharmacocyte-based form of FIX_{biot} is 5 to 10 times longer than that of the free form.

Material and Methods

The pharmacokinetics of FIX_{biot} was studied in 6 healthy volunteers (4 males and 2 females, aged 23–57 years, weight 50–70 kg) who had no previous episodes of hematological disorders and took no medication at least a week prior to the experiment. Two of them received intravenous infusions of FIX_{biot} (controls 1 and 2), while 4 others received intravenous infusions of autologous erythrocytes loaded with FIX_{biot} (volunteers A, B, C, and D).

The study was approved by local ethics committee of the National Center for Hematology, and written informed consent was obtained from each volunteer.

Material

Red blood cells were loaded with Aimafix D.I. 500 I.U. (FIX concentrate produced by Kedrion, S.p.A., Italy). According to the manufacturer's information, this product obtained in the form of lyophilized powder from human plasma is double virus inactivated. Its specific activity is 40 UI FIX per mg protein. One international unit (as defined by the WHO standard) corresponds to 5 μg of total FIX protein (both activated and not activated) in 1 ml of standard plasma. For the purpose of this study, FIX was biotinylated with a biotinylation reagent kit (Pierce, USA).

Reagents used in this study included mouse monoclonal antibody against human FIX (Enzyme Research Labs, USA); streptavidin-alkaline phosphatase conjugate (E-6603, Molecular Probes, Eugene, OR, USA); N-hydroxysulfosuccinimide (EZ-Link™ NHS-Biotin, Pierce, USA); dimethyl sulfoxide (DMSO; Serva, USA); $NaHCO_3$ (Merck, Germany); and 10 mM sodium phosphate buffered saline (pH 7.4) containing 2.7 mM KCl and 137 mM NaCl (PBS; Sigma, USA).

Phenylmethylsulfonyl fluoride (PMSF), 4-hydroxyazobenzene-2-carboxyl acid (HABA), D-biotin, avidin, tris-(hydroxymethyl)aminomethane base and hydrochloride (TRIZMA base and TRIZMA hydrochloride), Na_2CO_3, casein, and dimethyl formamide (DMFA) were also from Sigma (USA). Other reagents were chemically pure or analytical-grade domestic products.

Disposables used included dialysis bags with a molecular weight cutoff >12000 (Sigma, USA), 96-well EIA/RIA plates (Corning Costar, USA), and sterile Millipore filters (pore size, 0.22 μm; diameter, 13 mm; Millipore, USA).

Methods

Biotinylation of factor IX

The biotinylation procedure was essentially as described in [37,38]. Five vials of "AIMAFIX D.I. 500 I.U." were used to reconstitute FIX in 5 ml of deionized water. The reconstituted factor was dialyzed against 3 changes of PBS for 12 h at 4°C to remove excess salts. After the protein concentration in the dialyzate was adjusted to 2 mg/ml with PBS, 10 mg/ml N-hydroxysuccinimide biotin prepared *ex temporo* in dimethyl formamide was added to the dialyzate (10 μl/ml). The mixture was incubated at room temperature (20°C) for 1 h. Unbound modified biotin was removed by dialysis against 3 changes of PBS for 12 hours at 4°C. The protein was concentrated by centrifugation through a protein-impermeable membrane and then sterilized by filtration (pore diameter 0.22 μm). The final volume was about 3 ml; the protein concentration, as assessed with the Lowry method, was 5.8 mg/ml.

Determination of the extent of FIX biotinylation

The number of biotin molecules per biotinylated FIX molecule was estimated using the Pierce technique based on the ability of biotin to displace 4-hydroxyazobenzene-2-carboxyl acid (HABA) from its complex with avidin [39].

The $HABA_4$-avidin complex was obtained as follows: 440 μl PBS and 30 μl of 10 mg/ml avidin dissolved in deionized water were added to 530 μl HABA solution in 20 mM NaOH (2.4 mg/ml). The avidin concentration in the resulting canary-yellow $HABA_4$-avidin complex was 8 μM. The $HABA_4$-avidin reagent was stored at 4°C for no longer than 2 weeks.

Serial two-fold dilutions for all samples (and D-biotin standards 7.8 or 32 μg/ml) were carried out in 96-well flat-bottom plates using PBS. The final solution volume in each well was 100 μl. The $HABA_4$-avidin reagent (100 μl) was then added to each well, and the plate was placed on a shaker (400 rpm) for 10 min at room temperature. The $HABA_4$-avidin complex concentration was measured spectrophotometrically using a Bio-Rad Benchmark Microplate Reader (λ_{max}=500 nm, $\varepsilon_{500\ nm}$=34000 $M^{-1}cm^{-1}$). The biotin concentration in samples was determined from the calibration curve based on the biotin standard titration data.

The extent of biotinylation was calculated as the molar concentration of biotin divided by the molar concentration of protein, and was equal to 4:1 (approximately 4 biotin molecules per 1 protein molecule). The protein was assumed to be 100% FIX, with a molecular weight of 55000.

RBCs loading with biotinylated FIX

Stepwise dialysis was used to aseptically load RBCs with FIX_{biot} [29,30]. Red blood cells were isolated from citrate blood (blood-to-3.8% sodium citrate ratio of 9:1) and washed 3 times in sterile physiological saline by centrifugation (1250 g,

10 min). The resulting packed RBCs had a hematocrit of about 88%. Two ml of these packed cells and 0.8 ml of FIX_{biot} (diluted to a concentration of 5.8 mg/ml with PBS containing 5 mM glucose) were put into a dialysis bag presoaked for 4 h in the same buffer. The bag was immersed in a beaker equipped with a magnetic stir bar and filled with 200 ml of cold (4°C) hypoosmotic buffer containing 12.5 mM KH_2PO_4/KOH, 5 mM $MgCl_2$, 12.5 mM glucose, and 3.75 mM of adenosine triphosphate; pH 7.45. Dialysis was carried out at +4°C. Every 20 min, 60 ml of sterile distilled water was added into the external buffer. In total, 5 additions were made, which finally diluted the external buffer by a factor of 2.5.

The dialysis bag was then transferred into 200 ml of isoosmotic buffer (pH 7.45) preheated to 37°C and incubated at this temperature for 1 h to reseal the RBCs with FIX_{biot} inside. The isoosmotic buffer composition was as follows: 10 mM KH_2PO_4/KOH, 2 mM $MgCl_2$, 145 mM NaCl, 10 mM glucose, and 5 mM adenosine. The obtained RBC-carriers were put into a sterile test tube and washed 3–4 times in sterile physiological saline by centrifugation (1250 g, 10 min) until no trace of hemoglobin could be detected in the supernatant.

Study of pharmacokinetics of biotinylated FIX

Study subjects were given a single intravenous injection of FIX_{biot} either as a protein solution or as a suspension of carrier RBCs. Both the solution and the suspension were diluted 2-fold with sterile physiological saline immediately before injection. Venous blood samples (1 ml) were collected before and several times (up to 15 days) after the injection for monitoring of biotinylated FIX in plasma and RBCs.

Assays of biotinylated FIX in plasma and in RBCs

Preparation of plasma and RBC samples for analysis

Blood samples were centrifuged at 1250 g for 10 min. The supernatant plasma was collected and frozen at –32°C for subsequent analysis of FIX_{biot}. To assess the FIX_{biot} content in RBCs, they were washed in physiological saline to remove plasma trace levels and lysed. Specifically, packed RBCs (Htc 88%) were diluted 5-fold with deionized water, stirred, and lysed by freezing and thawing. The lysates were centrifuged at 12000 g for 10 min to remove cell membrane debris. Like plasma samples, the debris-free lysates were frozen until measurements were conducted.

Measurement of the biotinylated protein concentration

Biotinylated FIX in plasma samples, RBC lysates, and in the initial FIX_{biot} preparation (see above) were quantitatively assessed using a sandwich ELISA in the presence of the protease inhibitor PMSF at a final concentration of 1 mM.

Consecution of the procedures was as follows:

A. Addition of 100 µl per plate well of mouse monoclonal antibody against human FIX (diluted 1:1000 with 0.1 M carbonate-bicarbonate buffer; pH 9.4); overnight incubation at 4°C; rinsing with 0.1 M Tris-HCl (pH 7.5) (3 times, 200 µl per well).
B. Addition of 100 µl per well of 1% casein solution in the same washing buffer; incubation (2 h, 20°C); washing 3 times with the same buffer.
C. Addition of 0.1 M Tris-HCl (pH 7.5) (100 µl per each well of the plate).
D. Addition of the tested samples supplemented with 1 mM PMSF (100 µl) to the wells of the first row of the plate; standard titration of all samples (serial 2-fold dilutions); incubation (1 h, 20°C); washing of the wells with 0.1 M Tris-HCl (pH 7.5) (3 times, 200 µl per well).
E. Addition of 100 µl per well of streptavidin-alkaline phosphatase conjugate (diluted 1: 5000 in the same buffer); incubation (1 h, 20°C); standard washing 3 times.
F. Addition of 200 µl per well of 0.1% p-nitrophenyl phosphate in 0.2 M Tris-HCl buffer, containing 5 mM $MgCl_2$ (pH 9.5); incubation (15–20 min at 20°C).
G. Addition of 2 M KOH (100 µl per well), and reading of the optical density in the wells at 405 nm using a Bio-Rad Benchmark Microplate Reader.

RESULTS

RBCs loading with biotinylated FIX

Table 1 summarizes the results of RBCs loading with FIX_{biot} from 5 different experiments. The volume of obtained loaded cells (volume yield) averaged 55±10% of the cells volume at the start of the loading experiment. The FIX_{biot} concentration in lysates of the loaded cells was, on average, 24.3±10.5% of its initial concentration in the incubation suspension. The data are shown as the mean ± standard deviation.

Plasma concentration-time profiles after a single intravenous injection of biotinylated FIX solution

To have a control to compare with, FIX_{biot} was used first in the form of a solution. Plasma concentration-time profiles were obtained after a single intravenous injection in 2 volunteers. Specifically, 1 volunteer received 1080 µg of FIX_{biot} in 0.4 ml of physiological saline (control 1); and the other received 1160 µg, also in 0.4 ml of saline (control 2). The plasma level of FIX_{biot} was determined in blood samples taken at different intervals after the injection.

Figure 1A shows the plasma concentration-time profiles of FIX_{biot}. In both cases the plasma concentration of FIX_{biot} was maximal a short time after injection, decreasing thereafter. The time course of the disappearance of factor IX_{biot} from plasma is well described by a single exponent. This means that elimination of FIX_{biot} in this case follows the first-order kinetics. To estimate the elimination half-life, the data were replotted on a semi-logarithmic scale (Figure 1B). The $t_{1/2}$ averaged 8.85±5.6 h (Table 2).

Pharmacokinetics of RBC-loaded biotinylated factor IX

Four volunteers who participated in the pharmacokinetic study of RBC-loaded FIX_{biot}. Pharmacocytes (autologous RBCs aseptically isolated and loaded with FIX_{biot}) were adjusted to a hematocrit of 50% with sterile physiological saline and injected intravenously. The injected volume ranged from 1.5 to 2.4 ml, and the amount of FIX_{biot} injected was 210–400 µg (Table 2). Blood samples were taken at different intervals after the injection to monitor the FIX_{biot} content in the plasma and RBC fractions (Figure 2).

Table 1. Parameters of encapsulation for biotinylated factor IX ("Aimafix D.U. 500 I.U.") in red blood cells of different volunteers.

Volunteer[1]	V_0 [1], ml	V_{Pharm} [2], ml	C_0 [3], µg/ml	C_{Pharm} [4], µg/ml	Pharmacocytes yield		
					Volume[5], %	Concentration of entrapped protein in pharmacocytes[6],%	Total loaded protein[7], %
1 (A)	2.0	1.2	1246	400	60	32.1	19.3
2 (B)	2.0	1.0	1657	300	50	18.1	9.1
3 (C)	2.0	1.3	1657	260	65	15.7	10.2
4 (D)	2.0	1.2	1657	280	60	16.9	10.1
5	2.0	0.8	669	260	40	38.9	15.5
Average ± standard deviation (n=5)					55±10	24.3±10.5	12.8±4.4

[1] The initial volume of packed red blood cells used (ml); [2] The volume of obtained pharmacocytes (ml); [3] Concentration of factor IX_{biot} in initial incubation suspension (µg/ml); [4] Concentration of factor IX_{biot} in lysate of obtained pharmacocytes (µg/ml of pharmacocytes); [5] % of volume of initial red blood cells used; [6] % of protein concentration in initial incubation suspension; [7] % of total quantity of protein used for erythrocytes loading.

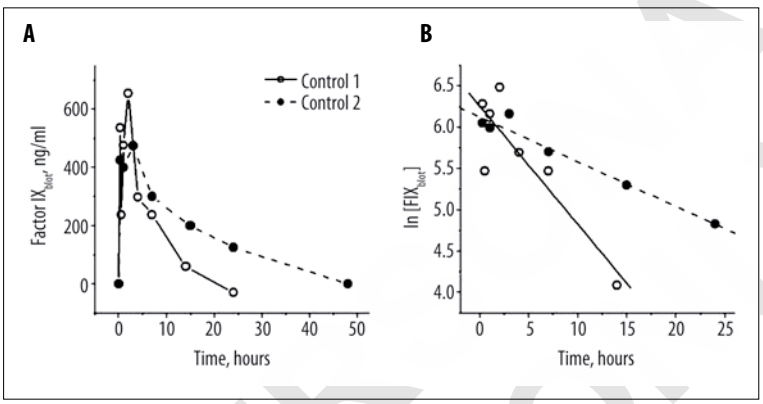

Figure 1. Plasma concentration – time profiles after a single intravenous injection of biotinylated FIX in solution to two healthy volunteers. Panel **A**: plasma concentration of biotinylated FIX plotted versus time; panel **B**: the same data presented on a semilogarithmic scale.

Calculation of the elimination half-time for RBC-loaded biotinylated factor IX

Insets in panels of Figure 2 present FIX_{biot} concentrations in RBC lysates plotted versus time on a semilogarithmic scale for the 4 volunteers studied. In all cases, the time course of FIX_{biot} could be described well with a single exponent. The results of calculations are summarized in Table 2.

The disappearance of FIX_{biot} from plasma did not follow the first-order kinetics. In some cases, its plasma concentration stayed at a constant level for up to 6 days, after which it began to decrease (Figure 2). However, even at the end of the experiment, it was still rather high (see Table 2).

Discussion

The concept of using erythrocytes as factor IX carriers appears attractive in terms of both life time increase and immune response decrease. However, several issues should be addressed before this can be carried out. First, factor IX should be loaded into erythrocytes with a sufficiently gentle method to obtain pharmacocytes with a long circulation time. Second, natural degradation of these erythrocyte carriers should maintain sufficiently high factor IX concentration in plasma. Finally, the loaded drug should retain its specific activity during the loading into cells and over the entire time of circulation in blood.

This study was focused on the first 2 problems. To monitor erythrocyte-loaded factor IX and to segregate it from the host factor IX, the preparation was biotinyilated prior to inclusion. This allowed us to quantitatively determine (using ELISA) small concentrations of the biotinylated protein in the presence of high background concentrations of naturally present factor IX. However, this does not allow segregation of activities of the naturally present and introduced factor IX. Earlier studies on inclusion of other enzymes (L-asparaginase [30], alcoholoxydase [40], glucooxydase and hexokinase [41], glucocerebrosidase [42], etc.) into erythrocytes suggest that factor activity would be retained. All those enzymes retained their activity inside the erythrocytes after the inclusion, and carried out their enzymatic function for a long time in the circulation. Retention of the factor IX activity should be investigated, but this should be a subject of another study employing different experimental approaches.

In our experiments, gentle stepwise dialysis was stably effective in loading biotinylated FIX into red blood cells (Table 1).

Table 2. Parameters of pharmacokinetic experiments.

Volunteer (weight, kg)	Total injected FIX_{biot}, µg	C_{max} of FIX_{biot}, ng/ml[1]		t_{max}, h[2]	C_{end} of FIX_{biot} in plasma, ng/ml (%)[3]	$t_{1/2}$, h	$t_{1/2}$ (mean ±SD), hours
		RBC	Plasma				
A (70)	400	145.6	42.5	360	5.31 (12.5)	94.2	73.9±16.0
B (70)	210	73.0	19.3	360	8.28 (42.9)	71.1	
C (50)	312	165.5	39.9	360	5.70 (14.3)	55.2	
D (50)	308	144.7	29.5	360	14.76 (50)	75.2	
Control 1 (45)	1080	–	655.0	24	0 (0)	4.9	8.85±5.6
Control 2 (60)	1160	–	475.0	48	0 (0)	12.8	

[1] Maximal concentrations of FIX_{biot} observed in RBC lysate and plasma samples during the pharmacokinetic experiment; [2] Total time of experiment; [3] Concentration of FIX_{biot} in plasma observed in the end of experiment (in parentheses the concentrations are given in % of maximal value observed for each volunteer's plasma during the study).

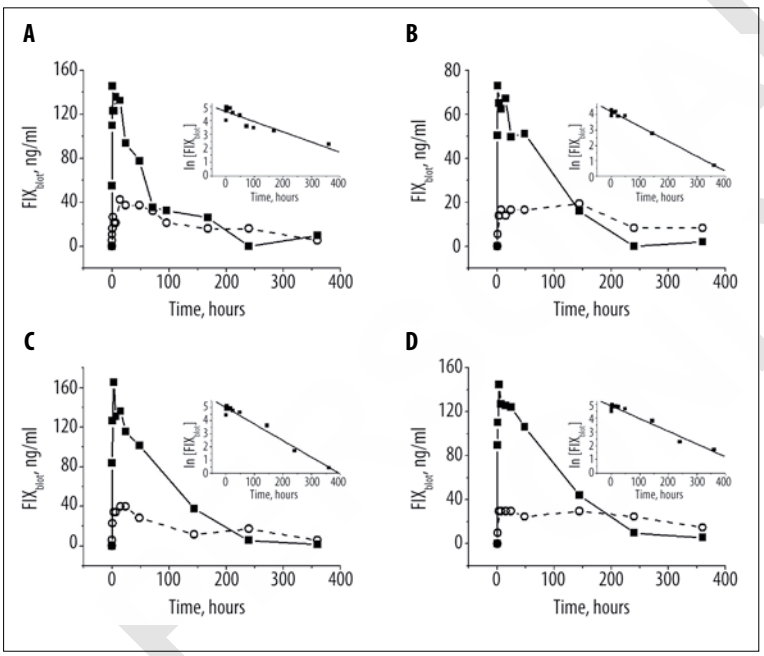

Figure 2. Biotinylated FIX in red blood cell lysates (■) and plasma (○) prepared from blood samples taken at different time after intravenous administration of FIX_{biot} loaded autologous cells (pharmacocytes) in four volunteers (**A, B, C and D**). The insets in the panels represent the same data for erythrocyte lysates on semilogarithmic scale.

The loaded red blood cells remained viable, persisting in the circulation for more than 15 days (Figure 2). Their sequestration obeyed the first-order equation, as judged by the linear plots of the logarithm of FIX_{biot} concentration in red blood cell lysates versus time (insets in Figure 2). The half-life of RBC-loaded FIX_{biot} in the circulation averaged 73.9±16.0 h (range: 94.2 to 55.2 h) (Table 2).

In the blood plasma of the volunteers who received FIX_{biot} in solution, the clearance half-life of FIX_{biot} averaged 8.85±5.6 h (Figure 1), shorter than that reported in the literature [43]. This experimental group was small and included only 2 volunteers, because we did not attempt to precisely determine the half-life time of FIX_{biot}. As there will be no biotinylation during treatment, our only goal was to ensure that biotinylation does not slow factor excretion, otherwise it would be difficult to interpret increase of $t_{1/2}$ upon infusion of erythrocyte-loaded FIX_{biot} as an increase in the factor life time due to its inclusion into cells. The measured half-life time for FIX_{biot} infused as a solution was even smaller than that reported previously for the non-biotinylated factor [43]. The observed discrepancy may be due to the fact that FIX was biotinylated, or may be due to the small size of the control group. However, we make a valid comparison of the clearance times for the free and RBC-loaded forms of FIX, because we used its biotynilated form in both cases. According to our data, in the circulation, the half-life of RBC-loaded FIX_{biot} was more than 8 times longer than the half-life of free FIX_{biot}.

Low-rate natural destruction of circulating pharmacocytes caused FIX_{biot} to be maintained at a sufficiently high level in plasma throughout the period of measurement of the pharmacokinetic curves (Figure 2). The maximal plasma concentration of FIX_{biot} averaged 25.1±3.7% of its maximum concentration in lysates of autologous red blood cells.

Even on day 15, the plasma concentration of FIX_{biot} was still 12.5–50% of the maximal (Table 2). Factor IX_{biot} entrapped in red blood cells disappeared from the circulation following the first-order kinetics. The mean maximal concentration of factor IX_{biot} in the erythroyte lysate observed during pharmacokinetic experiments was 75.5±3.6% (ranging from 71.6% to 80%) of the theoretical concentration based on the blood volume for each volunteer and assumption of a 45% hematocrit value. The clearance kinetics of plasma FIX_{biot} after injection of RBC-entrapped FIX_{biot} was different. In half of the cases, the plasma level of FIX_{biot} did not decrease over the first 6 days, suggesting that there was a continual leakage of the factor from the pharmacocytes undergoing gradual destruction in the circulation. Thus, the circulation life time of the pharmacocyte-based form of FIX is significantly prolonged compared with its free form.

The obtained results allow evaluation of the feasibility for our approach for creation of a novel drug form of factor IX (its economic efficiency). Analysis of Table 1 shows that inclusion of factor IX into erythrocytes by using stepwise dialysis allows for loading of ~12.8% of the initial quantity of factor IX used during inclusion. For the following estimations, we assumed that factor IX is infused in moderate hemophilia B approximately 3 times a week at a dose of 30 IU/kg [44], and that 1 IU is approximately 5 µg of factor IX. Then, over 2 weeks, a person weighing 70 kg will obtain factor IX 6 times in the quantity of 12600 IU, which corresponds to approximately 63 mg of factor IX protein. If the same quantity of factor IX is subjected to the inclusion procedure, then approximately 8 mg will be successfully loaded. Assuming normal blood volume of 4.9 l and hematocrit of 45%, we expect summary volume of erythrocytes of 2.2 l, and that of plasma 2.7 l. Based on the results of our experiments (Figure 2 and Table 2), it may be assumed that the maximal factor IX concentration in lysate of erythrocytes will be 75% of the theoretically possible (3.636 µg/ml), i.e., 2.727 µg/ml, and its maximal concentration in plasma will be 25% of the maximal lysate concentration, i.e., 0.682 µg/ml. If residual concentration after 2 weeks is ~25% of the maximal, it will be 0.170 µg/ml. This corresponds to ~3.4% of the normal factor IX concentration in the plasma of a healthy person. It has previously been shown that 5–10% of normal factor IX concentration is sufficient for normal coagulation [36]. Therefore, in order to obtain the same economic efficiency with the erythrocyte-loaded and free factor IX, the efficiency of factor loading should be increased at least 1.5-fold. This could be achieved by a modification of the method used, e.g., by continuously decreasing tonicity using a standard dialysis cartridge. Improvement of the drug loading method and the test of the drug activity during the inclusion and the following circulation should be the objectives of future studies.

Conclusions

In summary, we conclude that antihemophilic factor IX can be loaded into erythrocytes by a gentle stepwise dialysis method. The circulation life time of the erythrocyte-based form of FIX is significantly (5–10 times) prolonged compared with its free form. The natural destruction of loaded pharmacocytes provides a sufficiently high plasma concentration of factor IX. This suggests that RBC-based form of factor IX could be promising for clinical use. In order to make this drug form economically feasible, the efficiency of factor IX inclusion in the erythrocytes should be increased 1.5- to 3-fold.

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