Labeled Factor IX Kinetics in Patients With Hemophilia-B

By Kenneth J. Smith and Arthur R. Thompson

Labeled factor IX was infused five times into four patients with hemophilia-B. Ten-minute plasma recovery averaged 35% (SD ± 2) and the mean T½ β-phase elimination was 23 hr (± 5). No alteration in the postinfusion ^{125}I-factor-IX could be detected by radioautography of plasma samples run on polyacrylamide gels or on crossed-immunoelectrophoresis. Label was excreted into the urine as free ^{125}I-iodide. Kinetics were similar when the labeled preparation was infused alone or with a commercial concentrate containing unlabeled factor IX. Infusion of factor IX in man is best described by a two-compartment open pharmacokinetic model where factor IX is distributed in a space larger than the plasma volume.

ACCURATE PHARMACOKINETIC data on trace plasma proteins, such as factor IX, have been difficult to obtain. Previous survival studies have used clotting activity assays after infusions or following vitamin K antagonism or its reversal.[1-3] Postinfusion, activity disappears with an initial rapid phase followed by a slower decline, as first noted by Aggeler,[4] but recoveries and disappearance rates have been highly variable.[1] Despite structural homology, other vitamin-K-dependent factors have different recoveries and a wide range in survival.

Kinetic parameters can be more accurately estimated by infusing trace amounts of an isolated radiolabeled protein as shown with factor VIII (von Willebrand's factor).[5] We have investigated labeled human factor IX[6] infused into dogs with hemophilia-B and normal baboons showing comparable recoveries and survivals in these animals.[7] It was necessary to carefully characterize the labeled species to screen for any alteration that might affect its ability to circulate normally. These studies have been extended to examine the kinetics in human subjects with hemophilia-B. In humans, urinary excretion and the postinfusion plasma species were characterized.

MATERIALS AND METHODS

Materials were from the indicated sources: $Ag(NO)_3$, dithiothreitol, Sigma, St. Louis, Mo.; USP sodium citrate and NaCl, Mallinckrodt, St. Louis, Mo.; Na^{125} I, New England Nuclear, Boston, Mass.; acrylamide and bis-acrylamide, Bio-Rad, Richmond, Calif.; G-200 Sephadex, Pharmacia, Piscataway, N.J.; IgG Sorb, formalinized staphylococcal cell membranes, Enzyme Center, Boston, Mass.; factor IX concentrate (Konyne) and 25% normal serum albumin (human), Cutter Laboratories, Berkeley, Calif.; goat anti-rabbit IgG (P4,SA), Antibodies Inc., Davis, Calif.

Techniques

Clotting assays, gel electrophoresis, and isolation and radioiodination were as previously described.[6] Factor IX dose averaged 84 μg (range 20–120) from 5 labeling procedures on 4 separate preparations using 3 μg chloramine-T in a final volume of 100 μl for 10 sec. Labeled factor IX in 50 m*M* NaCl, 50 m*M* Tris (pH 7.5), 1 m*M* $CaCl_2$ was applied to 3.0 × 0.5 cm heparin-agarose columns[7] equilibrated and then washed with 10 ml of the same buffer. The column was then washed with 5 ml of 50 m*M* sodium citrate (pH 7.5), and factor IX was eluted with a 5-ml application of the citrate buffer containing 0.35 *M* NaCl. The 3–4 peak fractions (1 ml) were pooled and diluted with 2 volumes of sterile H_2O. Prior to injection, ^{125}I-factor-IX fractions were filtered through 0.22-μ Millex filters (Millipore, Bedford, Mass.) for sterilization. Filters were precoated by passage of 1 ml albumin. All preparations were nonpyrogenic on intravenous injection into 3 rabbits each (monitored 5 hr rectally) and sterile to 7 days thioglycolate broth incubations at both 22°C and 37°C.

Infusion Protocol

Four hemophilia-B patients (63–82 kg) who had antibody to hepatitis-B surface antigen volunteered for this study according to procedures approved by the Radiation Safety and Human Subjects Review Committees of the University of Washington and USPHS Hospital, Seattle. BUN, creatinine, and urinalyses were normal in all patients; transaminases were normal in patient 4, less than twice elevated in patients 1 and 2, and more than twice elevated in patient 3 at the time of study.

Two patients received crude commercial concentrate of factor IX 10 min prior to ^{125}I-factor-IX. Patient 1 was on home treatment with 1500 manufacturer's units of factor IX daily for a muscle hematoma; patient 3 received one infusion of 874 clotting units in his second study. In the other 3 studies, patients had not received therapy for at least 10 days. All patients took 3–5 drops saturated KI daily for 11 days starting 1 day prior to the study.

^{125}I-factor-IX was injected within 1 min with the syringe weighed prior to and following administration. The weight was used to calculate the injected dose. From 13 to 18 samples were drawn from a separate venipuncture site over the next 57–100 hr through a 19-gauge "heparin-lock" needle. Samples of 5 ml were drawn and divided into 2.5 ml vacutainer tubes containing EDTA or one-tenth volume 3.8% sodium citrate anticoagulants. Urine samples were collected at each void for up to 44–205 hr after infusion and timed volumes were measured; aliquots were stored at −20°C.

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Plasmas were centrifuged at 20,000 *g* for 20 min at 4°C to be rendered platelet-poor. For kinetic parameters, 1-ml aliquots of EDTA plasma were washed 4 times in 3 volumes of 20% trichloroacetic acid to precipitate protein and remove free iodide. Radioactivity was counted in a Nuclear-Chicago model 4233 gamma counter at 61% efficiency. Plasma radioactivity results were fit to a two-compartment open model with parameters determined from computer-derived, double exponent equations.[7] Plasma recoveries were determined as estimated plasma volume[9] multiplied by 10-min radioactivity divided by dose.

Characterization of the Postinfusion Species

Citrated plasmas were used. Barium citrate precipitates of 1-ml plasma aliquots were recovered by centrifugation following addition of 100 μl 0.15 *M* $BaCl_2$ and successive 1-ml washes with 0.15 *M* $BaCl_2$ and H_2O. Adsorbed protein was eluted in 200–300 μl of 0.15 *M* trisodium citrate (pH 7.5) and samples dialized for electrophoresis.[6]

Crossed-immunoelectrophoresis[8] was performed with 1.3% rabbit antifactor IX serum (100 Bethesda U/ml). For radioautography, dried polyacrylamide slabs or crossed-immunoelectrophoresis plates were placed in a standard x-ray cassette with a single intensifying screen (Cronex Lightning-Plus, Dupont, Wilmington, Del.) and double emulsion x-ray film (Xomat-R, Kodak, Rochester, N.Y.) for 4–20 hr at −70°C. Films were developed in a clinical x-ray processor. Gel filtration was through a 2.5 × 100 cm column equilibrated with 50 m*M* Tris (pH 7.5), 0.1 *M* NaCl.[7]

Urinary radioactivity was counted in 5-ml aliquots. AgI precipitates were obtained by mixing equal volumes of urine, H_2O, 2*M* HNO_3, and 2 volumes of 1% $AgNO_3$. This mixture was placed in a boiling H_2O bath for 2 min, and the resulting precipitate was washed twice each with 1 volume of water and 1 volume of acetone, prior to counting. Also, for urine samples, 1 mg/ml albumin was added prior to making the solution 20% in trichloracetic acid to screen for protein-bound radioactivity.

RESULTS

Preparation and Characterization of ^{125}I-Factor-IX

The mean specific activity was 0.83 (range 0.3–1.6) μCi/μg for 5 separate labeling procedures. Mean recovery of the labeled protein from heparin-agarose was 77% (range 54%–94%); recovery after filtration-sterilization averaged 59% (range 33%–87%). The radioactivity of each preparation was >95% adsorbed by barium citrate and >95% precipitated in 20% trichloroacetic acid.

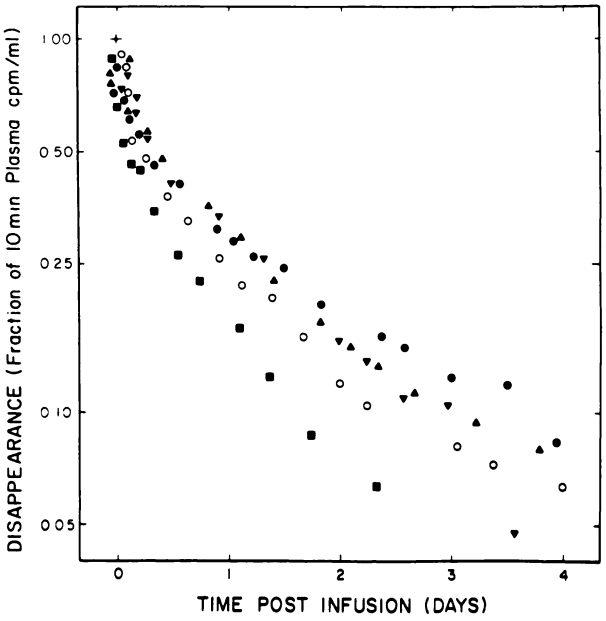

Fig. 1. Radiolabeled factor IX survival curves. Log fractions of 10-min levels versus time are shown. Symbols are for patients: 1 (▼); 2(▲); 3 without (○) and with (●) concentrate; and 4 (■).

On gel filtration, around 10% of the labeled protein eluted in the void volume with the rest of the material in the normal pre-albumin elution position for native factor IX. The void volume peak was seen whether or not labeled factor IX was applied with plasma and was present postinfusion for up to 17 hr. Both gel filtration peaks comigrated with native factor IX on polyacrylamide gels.

Pharmacokinetic Data

The five studies were quite similar, as shown in Fig. 1; calculated parameters are in Table 1. The only major difference between studies was a somewhat

Table 1. Pharmacokinetic Parameters* of Labeled Factor IX

Patient†	Dose Administered 10 min After Concentrate	Recovery (% Injected Dose)	Volumes: Central Distribution (Liters/kg)		Disappearance Half-Lives: α-Phase	Elimination (hr)	β-Phase	Clearance (ml kg^{-1} hr^{-1})
1 (11)	Yes	38	0.11	0.16	1.40	15	23	5.2
2 (21)	No	36	0.12	0.18	0.74	16	24	5.4
3 (24)	No	34	0.11	0.19	0.69	17	20	4.6
3 (24)	Yes	35	0.13	0.21	1.10	13	25	6.0
4 (26)	No	32	0.10	0.22	0.32	7	15	10.5
Mean (±SD)		35 ± 2	0.11 ± .01‡	0.19 ± .02	0.85 ± .41	14 ± 4	23 ± 5	6.3 ± 2.4

*From plasma activity time curves computer fit to a 2-compartment exponential equation;[7] for the 3-component analysis, central volume was identical and half-lives (in hours ± SD) were as follows: α, 0.6 ± 0.4; β, 9.6 ± 4.0; γ, 42 ± 14.

†Numbers in parentheses refer to data in Table IV, ref. 6, where baseline clotting and antigen levels were: 3 and 7, <1 and 17, <1 and 4, and <1 and 2 U/dl, respectively.

‡Central volumes from the mathematical model are nearly threefold greater than estimated plasma volumes.

shorter T½ β in the fourth subject; this patient was not available for a repeat study to determine if the results were peculiar to him or the preparation. Except for a slightly longer T½ α (patients 1 and 3), the kinetic parameters were unaffected by the presence of unlabeled factor IX.

When plasma radioactivity data were fit to a three-component exponential equation, significant decrease in residual mean squares was found for the first three, but not the last two, infusions (Table 1). Variability, particularly as indicated by the standard error for the estimates of the β-phase half-life, was markedly increased by the three-component treatment, however.

Urinary Radioactivity

No high molecular weight or protein-bound radioactivity could be demonstrated in the urine by gel filtration or trichloroacetic acid precipitation or after ultrafiltration through pressure dialysis membranes (PM-10, Amicon, Lexington, Mass.). The peak urinary excretion rate was delayed (8–25 hr) compared to the plasma disappearance, and the mean urinary elimination of radioactivity was 23% (SD ± 6) of the injected dose at 24 hr and 38% (SD ± 5) at 48 hr, consistent with excretion as free iodide.

The rates of decline in the urinary excretion rate with time were compared with the β-phase of the plasma disappearance curves. For subject 1, 2, and 4 (Table 1), the predicted β-phase elimination rates from the urinary data were 27, 26, and 18 hr, respectively; in good agreement with the 23, 24, and 15 hr, respectively, from their plasma data. In both infusions in patient 3, β-phase estimated from urinary excretion was twice as long as the plasma data. His skewed excretion of iodide is best explained by his moderately impaired renal function; his creatinine clearance at this time was 52 ml/min. Data for the second infusion in patient 3 are provided in Fig. 2B to be compared with the more typical results seen in patient 2 (Fig. 2A).

Postinfusion Characterization of Labeled Factor IX in Plasma

Plasma radioactivity was >95% precipitated in 20% trichloroacetic acid, and >90% of ^{125}I-factor-IX was precipitated with rabbit anti-factor IX using either goat anti-rabbit IgG serum or staphylococcal protein A membrane suspension. At 10 min postinfusion, 92%–96% of radioactivity adsorbed to barium citrate, but this fell to 77%–84% in samples obtained between 1.3 and 50 hr postinfusion. Label from plasmas or barium eluates migrated normally on gel electrophoresis (tested for up to 50 hr postinfusion in each study)

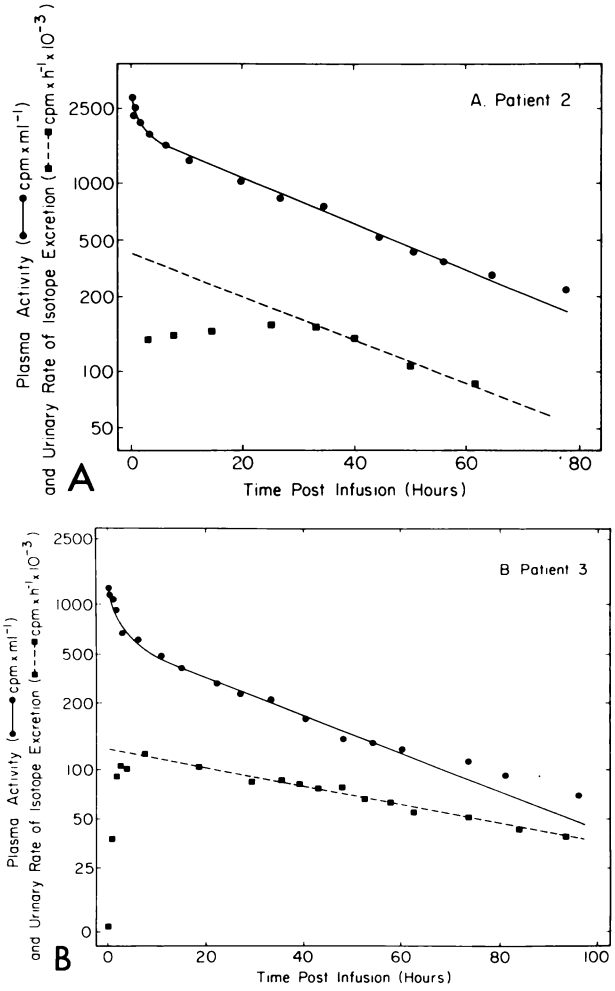

Fig. 2. Comparison of β-phase elimination rates by plasma disappearance and urinary excretion. Plasma disappearance is shown as trichloroacetic acid precipitatible radioactivity and urinary excretion rates as measurement of ^{125}I-iodide. The late decline of urinary excretion rate was used to approximate the late plasma radioactivity disappearance curve by calculating the slope of the log hourly excretion rate versus time and infusion.[10] (A) Data from patient 2 in which the disappearance half-life was calculated at 26 hr for urinary rate compared to 24 hr by plasma disappearance. Similar results were found in patients 1 and 4 (not shown). (B) Data on patient 3 following concentrate in which the β-phase elimination rate for plasma disappearance was 25 hr as compared to 54 hr by urinary excretion; this patient had moderately impaired renal function. The same effect was noted in his study without concentrate. Urinary data are plotted as counts per minute (cpm) excreted per hour (h), with values divided by 10^3 before plotting.

and peaks on crossed-immunoelectrophoresis were identical to start material (Fig. 3). All radioactivity was in the platelet-poor plasma.

DISCUSSION

Widely variable recoveries (20%–60%) and disappearance rates are typical of clotting activity studies.[1] This could reflect subject differences, assay measure-

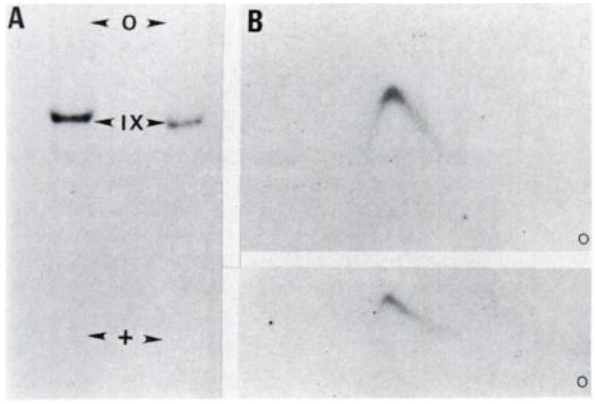

Fig. 3. Characterization of postinfusion plasmas by radioautography. (A) Polyacrylamide (7.5%) gel electrophoresis (Bio-Rad Model 220 Vertical Slab Gel Cell) in 0.1% sodium dodecyl sulfate of reduced 1.3-hr (left) and 12.5-hr postinfusion plasmas from patient 1 applied at origin (O). Labeled bands comigrate with isolated factor IX (IX); marker dye ran to the arrows at the anodal (+) end. (B) Crossed-immunoelectrophoresis (LKB Model 2117 Multiphor) of 3-hr postinfusion plasma of patient 1. Upper plate represents barium eluate and lower, unconcentrated plasma that corresponded in migration to uninfused start material (not shown). From the wells (O), anode is to the left for the first and top for the second dimensions. Barium citrate eluates of radioactivity (0.15 *M* sodium citrate) represented 60%–77% of plasma radioactivity.

ment error, and/or procedural variables such as the preparation, duration of infusion, and delayed or infrequent sampling. In contrast, kinetics based on radioactivity are quite similar among subjects. Except for a shorter $T^1/_2\alpha$, labeled data agree well with averages from clotting studies.[1,2] The more rapid initial clearance of labeled factor IX could be due to partial alteration of the protein, but it more likely reflects the frequent early sampling coupled with precise measurement. When the labeled studies are compared with those on normal baboons, kinetics were the same except for a somewhat longer human $T^1/_2\beta$ that is best explained by species differences in the factor IX.[7] In either species, the similarity of parameters when microgram quantities of labeled factor IX were given with or without therapeutic levels of unlabeled factor IX supports first-order kinetics. This suggests that factor IX recovery or survival will not vary with preinfusion levels or the amount of material infused.

After 50 hr postinfusion, plasma radioactivity deviated some from the log activity versus time curves. Low levels of counts were measured at these points and free trapped ^{125}I-iodide in the trichloroacetic acid precipitates could explain this finding. A second possibility is a minor contribution by a third compartment; this was not supported by urinary excretion rates, however.

Prothrombin represents the only other vitamin-K-dependent protein for which normal human kinetics of a radiolabeled species is available. Although it is nearly 20% larger and circulates at around a 25-fold greater concentration than factor IX, data from normal subjects fit a two-compartment open model.[11,12] Compared to factor IX, prothrombin had a greater plasma recovery and longer survivals; half-lives for α and β-phases of elimination were 12 and 73 hr, respectively.[12] First-order kinetics were observed in two hypoprothrombinemic patients before and after plasma treatment; for factor IX, the same lack of a dose-dependent survival was noted in normal baboons[7] and the current data in patients with hemophilia-B.

In human infusions, the labeled factor IX species behaved as native protein on polyacrylamide gel electrophoresis, gel filtration, and crossed-immunoelectrophoresis. With the kinetic parameter results, this is strong evidence that the labeled species, as infused, has not been altered in isolation or by radioiodination. Furthermore, radioiodinated factor IX, prepared by brief oxidation with chloramine-T, shows normal activation cleavage patterns.[13,14]

Potential future applications of in vivo infusions with labeled factor IX include the following: (1) physiologic relevance of contact activation versus any alternative pathway in intrinsic system activation; (2) pathogenesis of acquired factor IX deficiencies or disseminated intravascular coagulation; (3) survival of abnormal factor IX molecules isolated from hemophilia-B patients; and (4) survival of chemically altered factor IX proteins to select those that retain procoagulant activity with increased recovery or prolonged survival. The fourth consideration has potentially profound implications for the clinical management of patients with hemophilia-B.

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