Population Pharmacokinetics of Recombinant Factor XIII in Cynomolgus Monkeys

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ABSTRACT

Hemostasis in humans and other animals is a complex process that controls blood loss after a vascular injury. Factor XIII (FXIII) stabilizes clots primarily by cross-linking fibrin, thus protecting a newly formed clot from fibrinolysis by plasmin. Congenital deficiencies in humans involving FXIII are associated with delayed bleeding and wound healing and severe spontaneous hemorrhaging. These symptoms can be alleviated by intravenous administration of enriched FXIII plasma fractions. Circulating plasma FXIII is found as a heterotetramer that dissociates in the presence of calcium and thrombin into an active dimer and 2 inactive monomers. The recombinant FXIII under investigation is the active dimer alone. A 3-compartment, nonlinear population pharmacokinetic model was implemented in NONMEM V and then used to analyze data from preclinical studies in cynomolgus monkeys. The model simultaneously describes endogenous production of dimer ($0.622 \mu\text{g kg}^{-1} \text{hr}^{-1}$) and monomer ($12.1 \mu\text{g kg}^{-1} \text{hr}^{-1}$), and the administration of recombinant dimer. The model incorporates the rate and extent of complexation of recombinant dimer with available endogenous monomer ($6.59 \text{ mg}^{-1} \text{kg hr}^{-1}$) to form the heterotetramer. Half-lives for dimer, heterotetramer, and monomer (3.33 hours, 2.83 days, and 3.94 hours for A_2 , A_2B_2 , and B, respectively) were estimated, along with their variability in the population studied.

KEYWORDS: pharmacokinetics, modeling, FXIII, population kinetics

INTRODUCTION

Platelet-derived FXIII appears only in a dimer form, composed of 2 A subunits (protein-glutamine g-glutamyltransferase, E.C. 2.3.2.13).¹ Plasma-circulating FXIII is a heterotetramer, composed of 2 A subunits and 2 B subunits. The enzymatic activity of FXIII resides in the A subunit. The

B subunit acts as a carrier in plasma, extending the half-life of the heterotetramer (A_2B_2) compared with that of the dimer (A_2) or monomer (B) alone.² In the presence of activated thrombin and calcium ions, the plasma FXIII heterotetramer dissociates into 2 B subunits and the A_2 dimer. A_2 , then activated, acts as a transglutaminase to catalyze the formation of gamma-glutamyl-epsilon-lysine cross-links between fibrin molecules, stabilizing the fibrin clot. In addition, A_2 has been shown to cross-link alpha-2-plasmin inhibitor, fibronectin, to the fibrin alpha chains.³ Muszbek et al provided a recent overview of many of the details of this important protein.⁴

Lack of FXIII is a rare congenital deficiency disease in humans.⁵ Prolonged bleeding, delayed wound healing, severe spontaneous hemorrhage, and habitual abortion characterize the disease. Deficiency is categorized into 2 classes: type I deficiency, which is the lack of both the A and B subunits, and type II, which is the lack of the A subunit alone. Bone marrow is thought to be the primary site of A subunit synthesis,⁶ while the B subunit is synthesized in the liver.⁷ Current treatment strategies involve intravenous injection of concentrated plasma-derived A_2B_2 on a prophylactic basis. Recently, a recombinant form of FXIII A_2 dimer (rA_2) has been under investigation as an alternative therapy for FXIII deficient individuals.

OBJECTIVES

The objective of this work was to model the complex pharmacokinetics of intravenously administered rA_2 in cynomolgus monkeys. As the endogenous dimer (cA_2) was indistinguishable from the recombinant protein, it was necessary to account for this confounding factor. The formation of the heterotetramer complex was observed to be very fast (minutes) when compared with the half-life of the heterotetramer (days), and so some compromises in the complexity of the model were expected owing to sampling constraints. Finally, the model was required to be robust enough to support the use of multiple, complex immunological assays.

MATERIALS AND METHODS

The test article, recombinant human Factor XIII (rA_2 ; ZymoGenetics Inc, Seattle, WA) was supplied as 5.0 mL frozen isotonic solutions in 3% sucrose, 20 mM histidine, 200 mM

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glycine, 0.01% (wt/vol) polysorbate 20, pH 8.0, with a nominal concentration of active ingredient (rFXIII) of 5 mg/mL.

Study Design

Data from 2 preclinical studies were analyzed in this study. Preclinical data was gathered in *Macaca fascicularis* (cynomolgus) monkeys ($n = 4$ at each dose level), which were dosed intravenously once with 0.5, 1.0, or 5.0 mg/kg rA₂ (ZymoGenetics). Plasma samples were drawn at 0.25, 1, 2, 4, 8, 24, 72, 120, 168, 240, 336, 504, and 672 hours after dosing. Multiple-dose response studies were also performed in cynomolgus monkeys ($n = 6$ at each dose level), where 0.3, 3.0, and 6.0 mg/kg of rA₂ were administered intravenously once daily for 14 days. Plasma samples were drawn at 0, 0.25, 6, and 24 hours following the first dose on Day 1; predose and 15 minutes postdose on Days 2, 7, and 10; and predose, 0.25, 6, 24, and 48 hours postdose on Day 14. Treatment of animals was in accordance with regulations outlined in the United States Department of Agriculture (USDA) Animal Welfare Act (9CFR, Parts 1, 2, and 3) and the conditions specified in the Guide for the Care and Use of Laboratory Animals.⁸

Enzyme-linked Immunosorbent Assays

Total A₂ Enzyme-linked Immunosorbent Assays

A Total A₂ enzyme-linked immunosorbent assay (ELISA) was developed and validated to support pharmacokinetic measurements of FXIII A₂ in cynomolgus monkey plasma. The Total A₂ ELISA uses a polyclonal rabbit anti-FXIII A₂ subunit antibody to capture FXIII protein species from plasma ethylene diamine tetra-acetic acid (EDTA) samples. There are 3 species of FXIII that can be recognized by the anti-FXIII antibody in plasma:

1. rFXIII A₂ (recombinant human)(rA₂);
2. rFXIII A₂ (recombinant human) + cB₂ (endogenous monkey)(rA₂cB₂);
3. FXIII A₂ (endogenous monkey) + cB₂ (endogenous monkey)(cA₂cB₂);

The contribution of monkey endogenous FXIII A₂ to measurements of dosed rFXIII A₂ cannot be discerned by this assay. The same rabbit polyclonal anti-FXIII A₂ subunit antibody is biotinylated and used as a detection reagent. Purified rA₂ (ZymoGenetics) diluted into assay buffer was used as a standard for quantification of FXIII A₂ species. Streptavidin-horseradish peroxidase (HRP) and the substrate ortho-phenylenediamine dihydrochloride (OPD) were used for colorimetric detection of the FXIII A₂ concentration in standards, quality control samples (QCs), and test samples. Data from assay validation studies using QCs made from pooled cynomolgus monkey plasma consisting of both an endogenous level of A₂B₂ and endogenous A₂B₂

plus spiked rA₂ demonstrate the assay is accurate to within 20% of nominal concentrations (%RE), precise to within 15% coefficient of variation (CV) of measured concentrations, and sensitive to 0.79 µg/mL.

FXIII A₂B₂ Tetramer ELISA

An A₂B₂ ELISA was developed and validated to support pharmacokinetic measurements of FXIII tetramer (A₂B₂) present in cynomolgus monkey plasma. The A₂B₂ ELISA uses a polyclonal rabbit anti-FXIII B subunit antibody to capture the B subunit of the heterotetramer from plasma, and a polyclonal rabbit anti-A subunit antibody to detect the A subunit of the heterotetramer. The polyclonal is specific to the B subunit and does not cross-react with the A₂ portion of the molecule. The assay recognizes FXIII tetramers composed of rA₂ complexed to monkey B₂ (rA₂cB₂) as well as endogenous monkey cA₂cB₂. Purified rA₂B₂ (prepared by ZymoGenetics) diluted into assay buffer was used as a standard for quantification of rA₂B₂ and A₂B₂. Streptavidin-HRP and the substrate OPD were used for colorimetric determination of FXIII tetramer concentration in standards, QCs, and test samples. Data from assay validation studies using QCs made from pooled monkey plasma containing an endogenous level of monkey A₂B₂ demonstrate that the assay is accurate to 20% relative error (RE), precise to within 20% CV of measured concentrations, and sensitive to 1.5 µg/mL.

Free FXIII-B Subunit ELISA

A free B ELISA was developed and validated to support pharmacokinetic measurements of the free B subunit of FXIII present in human plasma. In principle, the free B ELISA does not detect the B subunit when it is bound in the A₂B₂ tetrameric state and is therefore specific for only the uncomplexed B subunit. The ELISA uses a donkey anti-mouse IgG to capture a monoclonal antibody specific for the free B subunit of FXIII. The detection antibody is a biotinylated polyclonal rabbit anti-FXIII B subunit antibody. Purified human B subunit diluted into assay buffer was used as a standard to quantify monkey FXIII B subunit. Streptavidin-HRP and the substrate tetramethyl benzidine (TMB) were used for colorimetric detection of the FXIII free B subunit concentration in standards, QCs, and test samples. Data from assay validation studies using QCs made of pooled monkey plasma containing an endogenous level of human free B subunit demonstrate the assay is accurate to within 20% RE, precise to within 25% CV of measured concentrations, and sensitive to 0.313 µg/mL.

Pharmacokinetic Model

It is assumed that the recombinant A₂ dimer (rA₂) and endogenous (cA₂) are functionally identical, are equivalent by ELISA methods, are equally capable of complexing with free

B monomer, and are kinetically identical. Each species is assumed to be eliminated from the plasma following mono-exponential decay, but species are modeled as having different apparent half-lives. We assume endogenous A_2B_2 is created by constant synthesis of endogenous cA_2 and B, release into the plasma, and subsequent association in the plasma to form cA_2B_2 . Intravenously administered rA_2 is expected to complex with the available endogenous circulating free B to form rA_2B_2 . The association rate of A_2 and B is very fast and is dependent on the concentration of both A_2 and B. To be completely correct, a form representing cooperative binding kinetics would model the association explicitly. However, this would increase the complexity of the model, and require intensive sampling early in the study to estimate this fast process. We do not expect dissociation of unactivated FXIII to play a role, and so this process is discounted. The binding constant, K_d , has been estimated to be 4×10^{-7} M, and free A_2 in the plasma has not been noted in any study.⁹ Graphically, we can depict this system as a 3-compartment model with nonlinear rate constants, as shown in Figure 1 (see Jacquez for a review of compartmental modeling¹⁰).

The following differential equations were used to describe the fate of the 3 molecular species:

$$\frac{d}{dt}A_2(t) = -1 \cdot [K_a \cdot B(t) + keA_2] \cdot A_2(t) + InfA_2 + D \cdot \delta(t) \quad (1)$$

$$\frac{d}{dt}A_2B_2(t) = +3 \cdot [K_a \cdot B(t) \cdot A_2(t)] - keA_2B_2 \cdot A_2B_2(t) \quad (2)$$

$$\frac{d}{dt}B(t) = -2 \cdot [K_a \cdot A_2(t) + keB] \cdot B(t) + InfB \quad (3)$$

where $A_2(t)$, $A_2B_2(t)$, and $B(t)$ are the masses of A_2 , A_2B_2 , and B, respectively, in the plasma as a function of time (mg kg^{-1}); K_a is the rate constant describing the association of A_2 and B ($\text{mg}^{-1} \text{kg hr}^{-1}$); keA_2 , keA_2B_2 , and keB are the first-order rate constants that describe the elimination of A_2 ,

A_2B_2 , and B, respectively, from the plasma (hr^{-1}); $InfA_2$ and $InfB$ describe the constant influx of endogenous cA_2 and B, respectively ($\text{mg kg}^{-1} \text{hr}^{-1}$); D is the bolus dose amount administered intravenously on a per body-weight basis (mg kg^{-1}); $\delta(t)$ is the Dirac delta function (hr^{-1}). It is important to note that the differential equation system is then expressed in terms of (mass protein)/(body-mass)/(time), since the dose is expressed per unit of body weight in these studies. The coefficients of 2 and 3 in the differential equations arise from the stoichiometry of the biochemical association: $1A_2 + 2B \rightarrow 1A_2B_2$. The tetramer differential equation contains the counter-intuitive constant “3,” which arises from summing the contribution of B (2) and A_2 (1). Maintenance of a constant A_2B_2 level in the plasma requires balancing endogenous production with protein turnover rates. Thus, the initial conditions of the system are completely specified by the kinetic rate constants and endogenous production rates, and can be determined by solving the system of differential equations at steady-state ($t = 0$):

$$A_2(0) = \frac{2 \cdot InfA_2 \cdot keB}{k_a \cdot (InfB - 2 \cdot InfA_2) + keA_2 \cdot keB + \psi} \quad (4)$$

$$A_2B_2(0) = \frac{3}{4 \cdot k_a \cdot keA_2B_2} \cdot [K_a \cdot (InfB + 2 \cdot InfA_2) + keA_2 \cdot keB - \psi] \quad (5)$$

$$B(0) = \frac{1}{2 \cdot K_a \cdot keB} \cdot [K_a \cdot (InfB - 2 \cdot InfA_2) - keA_2 \cdot keB + \psi] \quad (6)$$

$$\psi = [4 \cdot InfB \cdot K_a \cdot keA_2 \cdot keB + (K_a \cdot (2 \cdot InfA_2 - InfB) + keA_2 \cdot keB)^2]^{\frac{1}{2}} \quad (7)$$

Although each of the ELISA assays measure from the same plasma space, the capture and detection species have different affinities for their targets. Due to the fact that the total A_2 assay measures in A_2 -equivalents and the A_2B_2 assay in A_2B_2 - equivalents, there should be a 2:1 ratio of the estimates for volume of distribution of total A_2 vs A_2B_2 . However, an observed underestimate of existing A_2B_2 using the total A_2 assay contributes to a more pronounced difference in volume of distribution estimates than can be explained by our expected 2:1 ratio. This results in the need to use different scale factors (volumes of distribution). The resulting measurement equations are:

$$totalA_2assay = \frac{A_2(t) + A_2B_2(t)}{VtA_2} \cdot (1 + \varepsilon_1) \quad (8)$$

$$A_2B_2assay = \frac{A_2B_2}{VA_2B_2} \cdot (1 + \varepsilon_2) \quad (9)$$

$$freeBassay = \frac{B(t)}{V_B} + \varepsilon_3 \quad (10)$$

Figure 1. A diagram of the 3-compartment model that describes the kinetics of A_2 , A_2B_2 , and B as a function of time. The elimination rates are zero-order constants, and different for each species. The flux of A_2 and B to form A_2B_2 are nonlinear, depending on both A_2 and B amounts. The flux also follows the stoichiometry of the reaction: $1A_2 + 2B \rightarrow 1A_2B_2$.

where VtA_2 , VA_2B_2 , and VB represent the volumes of distribution that account for antibody affinity differences for the total A_2 , A_2B_2 , and free B assays, respectively ($L \cdot kg^{-1}$); each assay is assumed to have a measurement error that is proportional to the model for the measurement (total A_2 and A_2B_2 assay) or simply constant (free B assay). The free B assay was modeled in this manner due to the data set including very low values for this assay (zero in some cases). Under a proportional error model, these data would be thought of as very precise, and so they would be weighted heavily during the model-fitting process, resulting in skewed under-prediction of the baseline levels.

Population/Statistical Model

Population pharmacokinetics is the study of sources of variability in PK profiles in individuals comprising a population targeted for drug treatment. Physiological, pathological, genetic, allometric, and many other differences between subjects can vary the dose-concentration profiles between subjects. Population pharmacokinetics attempts to ascribe measurable differences between subjects, with the ultimate goal of tailoring a dose by those factors.¹¹ Several approaches have been suggested, but we focus on mixed-effects modeling as a framework to account for many sources of variability.¹²

Many of the parameters described so far are, by construction, weight normalized. The dosage levels per animal were also weight normalized. This simplified the development of a population statistical model, as a source of between-subject variability, body mass, was already accounted for. However, the elimination rate-constants were not weight-normalized, and it was necessary to add a random-effect to each of these parameters. Our subject-specific model parameters can then be mapped to parameters that do not change over the population (fixed-effects, μ) and parameters that change subject-to-subject (random effects, η):

$$\begin{array}{ll} \text{InfB} = \theta_1 & \text{mg} \cdot \text{kg}^{-1} \cdot \text{hr}^{-1} \\ \text{InfA}_2 = \theta_2 & \text{mg} \cdot \text{kg}^{-1} \cdot \text{hr}^{-1} \\ \text{VtA}_2 = \theta_3 & L \cdot \text{kg}^{-1} \\ \text{VA}_2\text{B}_2 = \theta_4 & L \cdot \text{kg}^{-1} \\ \text{VB} = \theta_5 & L \cdot \text{kg}^{-1} \\ \text{keA}_2 = \theta_6 \cdot e^{\eta_1} & \text{hr}^{-1} \\ \text{keA}_2\text{B}_2 = \theta_7 \cdot e^{\eta_2} & \text{hr}^{-1} \\ \text{keB} = \theta_8 \cdot e^{\eta_3} & \text{hr}^{-1} \\ \text{Ka} = \theta_9 & \text{mg}^{-1} \cdot \text{kg} \cdot \text{hr}^{-1} \end{array}$$

where model parameters Ka , InfB , InfA_2 , VtA_2 , VA_2B_2 , and VB are modeled as fixed-effects that do not vary across the population; the individual's model parameters keA_2 , keA_2B_2 , and keB are modeled as random effects whose values are influenced by their typical values across the population but can still vary subject-to-subject.

Finally, we must specify the distributional assumptions for the random variables we have introduced (η and ε). We assume independent, normal distributions for these random variables, and so each individual's random-effects can be modeled as:

$$\varepsilon_i \sim N(0, \Sigma_{ii}) \quad i = 1..3 \quad (11)$$

$$\eta_j \sim N(0, \Omega_{jj}) \quad j = 1..3 \quad (12)$$

where $N(\cdot)$ is the normal distribution; Ω is a diagonal, 3×3 matrix that describes the variance of the between-subject random-effects; Σ is a diagonal, 3×3 matrix that describes the variance of the within-subject (measurement error) random-effects.

Our population fixed-effects parameter vector that we wish to estimate is a combination of the fixed effects that appear in the individual's parameters (θ), the variance of the between-subject random-effects (Ω), and the variance of the within-subject random-effects (Σ), yielding 15 parameters of interest:

$$\alpha = [\theta_1, \theta_2, \theta_3, \theta_4, \theta_5, \theta_6, \theta_7, \theta_8, \theta_9, \Omega_{11}, \Omega_{22}, \Omega_{33}, \Sigma_{11}, \Sigma_{22}, \Sigma_{33}] \quad (13)$$

The model was implemented in NONMEM V, using the ADVAN8 subroutine (arbitrary stiff differential model) with a tolerance of $1E-8$. The PDx-Pop interface was used to access NONMEM functionality and produce plots and reports (GloboMax, Hanover, MD). The first-order approximation to the maximum likelihood was selected as the objective function, with the requirement of 3 significant digits in the solution. The differential equation solver was challenged by this stiff system of equations, and numerical issues precluded the use of more rigorous objective functions.

RESULTS

Estimates for the population fixed effects were computed in less than 1 hour on a desktop personal computer (Intel Pentium 4, 2.3 GHz). The "typical" subject (where the random effects are set to zero) predictions well accounted for the actual data for both the single- and multiple-dose studies at all dose levels. Moreover, if the random effect values for each subject were also included, the agreement (as expected) improved. Figure 2 shows the 1674 data points (y-axis) collected from all studies plotted against the typical prediction (top panel). A perfect agreement would generate x-y pairs on a unity line. Points far from the unity line indicated possible disagreement between the model prediction and data (goodness of fit). We can see some 20 points that fell far from the unity line, all in cases where the prediction was higher than the actual data. However, this subset represented $\sim 1\%$ of the total data. When we also compared the "individualized" fits to the actual data (where the effects specific for each subject were included), the overall goodness

Figure 2. Top panel: Plot of the single- and multiple-dose data (DV) vs the predicted “typical” population predictions (PRED). Bottom panel: Plot of the single- and multiple-dose data (DV) vs the individualized predictions (IPRED). On each graph (DV) vs the respective prediction, 1674 data points are plotted, and perfect agreement between these values would generate x-y points lying along the unity line (solid line).

of fit was improved (Figure 2, lower panel). There remained a group of data points where the model over predicted, but the number was fewer. The problem can be traced to the washout observation period of the tetramer in the multiple-dose trial. The elimination rate of the tetramer appeared to be faster than in the single-dose trials, suggesting that the elimination kinetics may be more complicated than the assumed first-order profile. However, the tetramer levels that accumulated during the multiple-dose trials were much higher than normal physiological levels (and therefore outside of projected clinical relevance), so it appeared sufficient for our purposes to retain the kinetic structure that described typical physiological conditions.

Reviewing goodness of fit in this way is useful in capturing the overall quality of the predictions by distilling the volume of data into a concise set of plots. We now expand the volume of these goodness-of-fit metrics to partition the data by assay (total A_2 , A_2B_2 , and free B) and dose level. The doses were split into low, medium, and high (0.5, 1.0, and 5.0 mg/kg for the single-dose and 0.3, 3.0, and 6.0 mg/kg

for the multiple-dose trial) doses, giving us a sense of how the model fit behaved over a 10- or 20-fold range of doses. In Figures 3, 4, and 5 (total A_2 , A_2B_2 , and free B, respectively), the dose levels index the columns. The first row of these figures shows the data value (DV) against the “typical” population predictions (PRED, plusses) and individualized predictions (IPRED, circles). The second row of these figures shows the weighted residuals (WRES) of the single- (plusses) and multiple-dose (circles) data and fits vs time. The third row of these figures shows the DV and PRED vs time for the single-dose study, while the fourth row displays the same information for the multiple-dose study. Typical population predictions were connected by lines as a guide for the eye, not by simulated model values, so care should be taken when interpolating between measured time points.

Beginning with the total A_2 assay (Figure 3) population and individual fits (top row), we can see that the model generally agreed with the data, except at very low and very high measurements (DV). The weighted residuals (Figure 3, second row), which were separated into single-dose (plusses) and multiple-dose (circle) groups indicated that the model systematically under-predicted the multiple-dose measurements and over-predicted the single-dose measurements. The third and fourth (single- and multiple-dose, respectively) rows in Figure 3 show the typical fit (solid line) and measurements (plusses) vs time, which confirmed this disagreement. However, the model had no provision for differences between the 2 studies. Possible rationales will be explored in the Discussion section.

Figure 4 shows the same information for the A_2B_2 assay. Here however, the typical and individualized fits (top row) appeared in very good agreement. The residuals indicated a slight under prediction for the single-dose study and a slight over prediction for the multiple-dose study (second row from the top). This was a reversal of the pattern seen for the total A_2 assay. Again, the overall fits can be seen for the single- and multiple-dose studies (Figure 4, third and fourth row). The model could not reconcile the apparent rapid elimination of the heterotetramer in the multiple-dose study (Figure 4, fourth row, third column) and the much slower elimination in the case of the single-dose study (Figure 4, third row, third column). Again, the model had no provision for differences between studies. Here, however, there was the possibility that the elimination of the heterotetramer was not monoexponential.

Finally, Figure 5 shows the same information for the free B assay. The weighted residuals showed some discrepancy in the multiple-dose fit, and the baseline free B levels were under-predicted in the multiple-dose studies. In general, however, the fits were in good agreement with the experimental data.

Once we were reasonably confident in the goodness of fit for the model, we turned our attention to the parameter estimates gained from the data. Table 1 lists the fixed effects, or parameters that do not change over the population,

Figure 3. Summary plots for the total A2 assay. Predictions are linearly interpolated and were calculated only at time points where samples were available.

estimated from the data. The meanings of the parameters are complex, and so we divide the results into 3 sections, following the division detailed in the Methods section.

A typical subject in the population studied would assume PK parameters (θ) equal to those in the first section of Table 1. The estimates were well defined, having a

Figure 4. Summary plots for the A2B2 assay. Predictions are linearly interpolated and were calculated only at time points where samples were available.

percentage relative standard error (%RSE) no greater than 19.7%. The endogenous production rates, here modeled as constant influxes into the plasma space, have not

been reported in the literature to date and were $0.622 \mu\text{g kg}^{-1} \text{hr}^{-1}$ for the dimer and $12.1 \mu\text{g kg}^{-1} \text{hr}^{-1}$ for the monomer. The elimination rate constants translated

Figure 5. Summary plots for the free B assay. Predictions are linearly interpolated and were calculated only at time points where samples were available.

easily to half-lives of 3.33 hours, 2.83 days, and 3.94 hours for A_2 , A_2B_2 , and B, respectively. The volumes of distribution were 142 mL, 32.7 mL, and 209 mL for

VtA_2 , VA_2B_2 , and VB, respectively, and can be compared with blood volume in a normal cynomolgus monkeys ($\sim 3.5 \text{ kg} = 270 \text{ mL}$).

Table 1. Parameter Estimate Results From Single- and Multiple-dose rA₂ Cynomolgus Monkey Studies*

Parameter	Estimate	%RSE	Units	
PK parameter (θ)				
InfB	0.0121	8.76%	mg kg ⁻¹ hr ⁻¹	
InfA ₂	0.000622	17.00%	mg kg ⁻¹ hr ⁻¹	
VtA ₂	0.0407	3.93%	L kg ⁻¹	
VA ₂ B ₂	0.00934	3.62%	L kg ⁻¹	
VB	0.0598	12.30%	L kg ⁻¹	
keA ₂	0.208	9.09%	hr ⁻¹	
keA ₂ B ₂	0.0102	10.50%	hr ⁻¹	
keB	0.176	15.90%	hr ⁻¹	
Ka	6.59	19.70%	mg ⁻¹ kg hr ⁻¹	
Population variability (Ω)				BSV
Var[keA ₂]	0.0629	137%	% ²	25.10%
Var[keA ₂ B ₂]	0.0268	38.10%	% ²	16.40%
Var[keB]	0.151	44.50%	% ²	38.90%
Assay variability (Σ)				RUV
Var[total A ₂ assay]	0.0730	16.40%	% ²	27.00%
Var[A ₂ B ₂ assay]	0.0751	15.70%	% ²	27.40%
Var[free B assay]	0.0857	19.50%	mg ² L ⁻²	0.293 mg L ⁻¹

*The fixed effects appearing in the PK model equations [PK parameter (θ)] are shown first in this table, along with their estimates and percentage coefficients of variation (%RSE = percentage relative standard error = standard error of the estimate/estimate mean $\times$ 100%) and units. These can be thought of as “typical” values in the studied population. The fixed effects that describe the between subject variability of the elimination rate constants [Population variability (Ω)] have units of percentage squared and are shown next with their estimates and precision. The between-subject variability (BSV) = (estimate mean of the variance)^{1/2} $\times$ 100% is reported here in the last column. The fixed effects that describe assay variability [Assay variability (Σ)] are shown last in this table, with their estimates and %RSE. The total A₂ and A₂B₂ assay errors were modeled as being proportional to the model prediction, and so the proportionality constant was estimated and reported here as a unit-less variance. The residual, unexplained error in the measurements (RUV) = (estimate mean of the variance)^{1/2} $\times$ 100% is reported in the last column. In contrast, the free B assay error was best modeled as being additive and independent of the model prediction, and so the estimate of this variance is expressed here as the square of the measurement units. The residual, unexplained error in this measurement RUV = (estimate mean of the variance)^{1/2} is also reported, now in the units of the measurement.

The second section of Table 1 lists the between-subject variability for parameters (Ω) that we have modeled as changing from subject to subject. The elimination rates, which were not normalized by weight, were modeled as varying subject-to-subject, and the variances were found to be 0.0629, 0.0268, and 0.151 for keA₂, keA₂B₂, and keB, respectively. The uncertainty of these parameter estimates was higher than for the typical parameter values, still being, at worst, a %RSE of 137%. Parameters relating to the fate of the recombinant dimer (keA₂, Ka) indicated that these reactions were very fast, and difficult to estimate given the sampling schedules used in these studies. We can also express the degree of influence subject-subject variability may have on these parameters: how much might any one individual in the population deviate from the “typical” population value? The square root of these variances $\times$ 100% yields the between-subject variability (BSV) of the PK parameters, which were reported in Table 1 as 25.1%, 16.4%, and 38.9% for keA₂, keA₂B₂, and keB, respectively.

The final section of Table 1 reports the estimated variability (Σ) of the assays. In the case of the total A₂ and A₂B₂ assays,

the measurement error was modeled as being proportional to the model value. The (unit-less) variances of the measurement error for these assays were found to be 0.0730 and 0.0751, respectively. The free B assay measurement error was modeled as being constant across the range of the assay, and the variance of the error was found to be 0.0857 mg² L⁻². These estimates can again be expressed relative to the assay values, yielding residual, unexplained variation (RUV) in the assays of 27.0%, 27.4%, and 0.293 mg L⁻¹.

DISCUSSION

Complex, noisy assays in biomedical experiments are common, but remain problematic. First, the noise level, if not properly modeled, can degrade parameter estimate quality. Second, the complexity can prevent unambiguous identification of a parameter of interest. Here, the total A₂ assay, sensitive to A₂B₂ and A₂, confounds the estimation of 2 elimination rate constants (keA₂ and keA₂B₂). Third, the sampling schedule may not be suitable for estimating all the parameters of interest.

The model included no provisions for differences between studies. There are 2 possible sources of differences that we have considered, but could not sufficiently justify for inclusion in our model. First, there is the possibility that the 2 populations of cynomolgus monkeys are truly different. The animals were all of Chinese origin, and both studies were performed at the same laboratory. Gender distribution was approximately equal between studies. Second, there is the possibility that some of the processes controlling this system are more nonlinear than we propose here. The formation of the heterotetramer and possible attenuation of the production rates will be examined later, and for the moment we will focus on the possibility that the elimination of the species (here, modeled as monoexponential decay) is influenced by the dose and regimen. The A_2B_2 elimination shows the largest deviation by exposure (Figure 4, third column, third and fourth rows). The single-dose prediction falls below the data, indicating that the elimination rate is too fast (third row), while the multiple-dose prediction falls above the data after the final dose (fourth row), indicating that the elimination rate is too slow. The total concentration for the heterotetramer in the multiple-dose trial is 4-fold higher than in the single-dose trial, so we can speculate that the assumption of a constant elimination rate constant may not hold across this range of exposures.

The endogenous production of A_2 and B were described as simply as was plausible as constant infusions into the plasma. The relative production rates indicate that the production of the B monomer is being maintained at a rate 20-fold greater than the production of A monomer. This agrees well with the observations that uncomplexed A_2 dimer is not observed in plasma in primates and man, and that there is an equal amount of uncomplexed and complexed B in plasma in man.² No attenuation mechanism was modeled, although we explored the possibility that A_2 and B production were regulated by the concentrations of each of the species, as has been suggested previously.¹³ In these cases, identifiability issues appeared, as the elimination rates could be adjusted with the attenuation to yield any number of possible solutions. Radio-labeled exogenous species could be introduced in new studies to determine the elimination rates independently.

Nonlinearities in the dynamics of the system are introduced in the simplest possible manner by making the flux of A_2 and B to A_2B_2 dependent on the counterpart species. Thus, an overwhelming amount of A_2 with nearly no B present, as is the case with higher doses after all the available free B is complexed, will result in very little further production of tetramer. The multiple-dose A_2B_2 assay data also shows a lack of superposition, which can be explained by noting that the free B level does not recover to the same amount just before the next dose. With less free B available to form a complex, less A_2 is complexed. Uncomplexed A_2 has a relatively shorter-half life, meaning excess dimer is quickly

eliminated. Knowledge of the association rate of the tetramer (Ka), previously only informed indirectly by in vitro binding affinity experiments, is stymied by infrequent sampling early in the experiment. From what is known about the heterotetramer formation, it may be more exacting to model the association as a cooperative binding process involving many binding states. However, intensive sampling early in the experiment, at the very least, would be required to identify the many parameters that would arise from including this level of detail.

Where it was appropriate, population modeling was applied to include between-subject variability within the model. Weight is an important covariate, and many parameters within the model were normalized by weight due to the dosing strategy. The elimination rate constants were not weight normalized, and so here we included random-effects. Estimates from these studies show that between-subject variability is present and is appreciable compared with the central tendency of the parameters. One measure of impact, BSV, shows that one standard deviation of the between-subject variability can be equal to 16% to 40% of the parameter value. Moreover, the estimate for the elimination rate of the dimer is very imprecise (%RSE = 137%). We did initially attempt to fix the association rate constant (Ka) to a very fast value, but this is a somewhat unsatisfactory assumption.

Finally, we believe this model may be useful as a prospective tool for simulating other dosing regimens. Figure 6 shows single- and multiple-dose experiments simulated using the typical population parameter values. We can ignore the assay variability and begin to get a sense of how the species are interacting. This is key, as the interaction is nonlinear in nature. Moreover, the complex immunological assays obscure the actual amounts of each of the individual species. The model can be used to simulate the expected actual amount of the species present in the blood both before and after exogenous administration.

CONCLUSIONS

The nonlinear mixed-effects model we have described was conceptualized from the basic stoichiometry of the prodrug formation: $1A_2 + 2B \rightarrow 1A_2B_2$. The initial data that was collected showed that the recombinant protein had an important interaction with the endogenous protein present in the blood. Therefore, we sought a model that could perform the following:

- seamlessly include endogenous production of native proteins as well as exogenous administration of exogenous recombinant proteins
- incorporate complex, noisy immunological assays that did not discriminate between endogenous and exogenous A_2 dimer

Figure 6. “Typical”/population predictions for a single bolus of 5 mg/kg rA_2 (top panel) and multiple boluses of 6 mg/kg rA_2 (bottom panel) administered daily for 14 days. The time axis (x-axis) is extended to include 100 hours of predose baseline levels detected by the assays. The A_2B_2 assay is shown as a solid line, while the total A_2 assay is shown as a broken line. The free B assay levels are so low as to not show well on these scales, but a solid line can be seen near 1.0 on the y-axis that drops to near zero, while uncomplexed rA_2 is present just after dosing.

- capture very fast (seconds: heterotetramer complexation) and very slow (days: heterotetramer elimination) dynamic processes
- explain the nonlinear dose–response relationship between rA_2 administration and rA_2B_2 formation
- simulate other dosing regimens

The model developed describes the PK profile of recombinant FXIII dimer when administered to cynomolgus monkeys. Between-study differences, although not understood, were reasonably minor and did not require modifications to the model. At the high dose levels, the data began showing signs of deviating from the model predictions, indicating that some of the assumptions underlying the model were not valid across such a wide exposure range. However, looking forward to the use of the model in the future, we expect that the range of clinical exposure (as is usually the case) will not be as dramatic as seen in this animal model.¹⁴

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