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Research Paper

An Assessment of Recombinant Human Erythropoietin Effect on Reticulocyte Production Rate and Lifespan Distribution in Healthy Subjects

Wojciech Krzyzanski,[1] and Juan Jose Perez-Ruixo[2,3,4]

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Purpose. An empirical pharmacodynamic model was developed to assess the effect of recombinant human erythropoietin (rHu-EPO) treatment on the reticulocyte production rate and lifespan distribution.
Materials and Methods. Single doses of rHu-EPO at levels 20, 40, 60, 90, 120, and 160 kIU were administered to healthy volunteers ($n = 8$ per dose level). Erythropoietin plasma concentrations as well as hematologic responses were measured up to 42 days. The hematological data were used to determine explicit relationships between reticulocyte and red blood cell counts (RBC) and the reticulocytes' production rate and lifespan distribution.
Results. The parameter estimates obtained by simultaneous fitting of the model to the reticulocyte and RBC data revealed that rHu-EPO transiently increased the reticulocyte lifespan from the baseline value of 1.7 days to 3.4 days and the effect lasted for 8.3 days. The dose dependent increase in the reticulocyte production had the maximal value of 77.5 10^9 cells/l/day and was followed by a rebound that was less than 9% of the baseline value. Both reticulocyte and RBC responses were preceded by a dose-independent lag time of 1.7 days.
Conclusions. The effect of rHu-EPO on the reticulocyte production rate and lifespan distribution was characterized. The results of the present study can be further utilized in building more mechanistic pharmacodynamic models of rHu-EPO stimulatory effects.

KEY WORDS: precursor–successor relationship; pharmacodynamic model; lifespan; recombinant human erythropoietin; NONMEM.

INTRODUCTION

Recombinant human erythropoietin (rHu-EPO) has been indicated for treatment of renal failure anemia as well as anemias induced by chemotherapy of cancer and AIDS patients. RHu-EPO stimulates production of red blood cells (RBC) by binding to erythropoietin receptors expressed on progenitor cells in bone marrow and initiating intracellular signaling pathways leading to inhibition of cell apoptosis, and enhancement of proliferation and differentiation (1). The stimulated progenitor cells differentiate to erythroblasts, which mature to reticulocytes. The reticulocytes are released to blood where they continue the maturation process to RBC. The RBC are removed from the circulation mostly due

[1] Department of Pharmaceutical Sciences, University at Buffalo, 547 Cooke Hall, Buffalo, New York 14260, USA.
[2] Clinical Pharmacology, Johnson & Johnson Pharmaceutical Research & Development, Division of Janssen Pharmaceutica, Turnhoutseweg 30, Beerse, 2340, Belgium.
[3] Pharmacy and Pharmaceutics Division, Department of Engineering, Faculty of Pharmacy, Miguel Hernández University, San Juan de Alicante (Alicante), Spain.
[4] To whom correspondence should be addressed. (e-mail: jperezru@prdbe.jnj.com)

ABBREVIATIONS: $\chi(t)$, Jump function; C_{EPO}, rHu-EPO plasma concentration; CF, Conversion factor for reticulocytes; $\Delta k_R(t)$, Change of $k_R(t)$ from the baseline value; Δk_{R1}, Increase in reticulocyte production rate due to rHu-EPO; Δk_{R2}, Decrease in reticulocyte production rate following exposure to rHu-EPO; $\Delta RET(t)$, Change of the reticulocyte count from the baseline value; $\Delta RBC(t)$, Change of RBC count from the baseline value; ΔT, Increase in the reticulocyte lifespan due to rHu-EPO treatment; ΔT_1, Duration of the rHu-EPO effect on reticulocyte lifespan distribution; ΔT_2, Duration of the rebound in reticulocyte production; $\delta(\tau)$, Dirac delta function; D, Dose; ε, Residual error; E, Effect; E_0, Baseline effect; E_{max}, Maximal effect; ED_{50}, Dose eliciting 50% of the maximal effect; $INT(z)$, The integral part of the number z; $k_{out}(t)$, Reticulocyte elimination rate; $k_R(t)$, Reticulocyte production rate; k_{R0}, Baseline reticulocyte production rate; k_{R1}, Reticulocyte production rate during rHu-EPO stimulation; k_{R2}, Reticulocyte production rate during the rebound; $\ell(t,\tau)$, Probability density function for reticulocyte lifespan distribution at time t; $\ell_0(\tau)$, Time independent probability density function for reticulocyte lifespan distribution; MRBC, Mature red blood cells; mature red blood cell count; $MRBC_0$, Baseline mature red blood cell count; N, Integer part of the ratio t/T_{RET0}; p.d.f., Probability density function; RBC, Red blood cells; RBC_0, Baseline red blood cell count; RET, Reticulocytes; reticulocyte count; RET_0, Baseline reticulocyte count; RHu-EPO, Recombinant human erythropoietin; σ, Standard deviation of the residual error; τ, Reticulocyte lifespan; T_0, Lag time between rHu-EPO administration and its effect; T_1, Time at which the effect of rHu-EPO on the reticulocyte lifespan distribution stops; T_2, Time at which the rebound ends; T_{RET}, Reticulocyte lifespan; T_{RET0}, Baseline reticulocyte lifespan; T_{RBC}, Red blood cell lifespan; t_{max}, Observed reticulocyte count peak time; $UIR(t)$, Unit impulse response.

to senescence. A single subcutaneous dose of rHu-EPO of 450 IU/kg administered in healthy adult humans causes an increase in reticulocyte count with a peak of 3.5% on day 7 with a lag time of 3 days (2).

A wealth of mathematical models has been developed that describe hematological responses (reticulocyte and RBC counts, hemoglobin blood concentrations, hematocrit) to rHu-EPO treatment in healthy subjects (3,4) and various patient populations (5,6). The stimulatory effect of rHu-EPO on reticulocyte, RBC, and hemoglobin production has been modeled by means of the Hill function of the rHu-EPO serum concentration whereas the loss rates of these hematological responses were controlled by the reticulocyte and RBC life spans. The lifespan, maturation, or transition times in those models have been considered dose independent. Flow cytometry analysis of reticulocyte RNA content indicates that the age distribution for reticulocytes released to blood due to stimulated erythropoiesis differs from that of normal reticulocytes (7). A pharmacodynamic model of the changes in the reticulocyte subtype distributions in stress erythropoiesis has been proposed (8). Consequently, rHu-EPO treatment affects not only the production rate of the hematological responses but also the values of the pharmacodynamic parameters controlling their loss rates.

The precursor–successor relationship holds between two compartments of a biological system if a known part of the material from the precursor is transferred to the successor. If there is no intermediate compartment, then the precursor is absolute (9). The precursor–successor relationship is known if the time course of the amount in the successor compartment can be determined from the time course of the amount in the precursor compartment. The basic tenet of such relationship is conservation of mass. Any deviation from it indicates mass "leakage." A physiological example of an absolute precursor–successor system is reticulocytes and mature erythrocytes. Assuming that each reticulocyte matures to an erythrocyte, the reticulocyte compartment constitutes the absolute precursor for the RBC compartment. Establishing a precursor–successor relationship between these compartments will allow one to predict RBC count at any moment of time solely from the reticulocyte counts for any stimulus of the reticulocyte production that preserves the "mass balance" between reticulocytes and mature RBC.

Most mathematical models describe the conversion of reticulocytes to mature RBC as a first-order process (10). The precursor–successor relationship between reticulocytes and mature RBC derived for such processes requires knowledge of the reticulocyte elimination rate constant that is difficult to obtain from the reticulocyte count data without employing modeling techniques. We have introduced another description of the reticulocyte conversion rate where the reticulocyte lifespan determines the transition rate to mature RBC. According to this concept a reticulocyte becomes a mature RBC after reaching a common population age (11). Such an age defines the reticulocyte residence time in the circulation and can be identified with a reticulocyte lifespan. This lifespan determines the reticulocyte conversion rate by a simple delay of the production rate. When stimulated by a therapeutic agent administered as a single dose, the reticulocyte lifespan can be easily identified from the reticulocyte vs. time plot as the peak time (11). We applied this concept to derive a precursor–successor relationship between reticulocytes and RBC.

Our objectives for the present study were (1) to derive a precursor–successor relationship between reticulocyte and mature RBC, (2) determine if rHu-EPO treatment affects reticulocyte production rate and lifespan distribution, and (3) quantify rHu-EPO effect on the time course of the reticulocyte production rate without reference to the serum concentrations. To address our objectives we used hematological data from healthy subjects who received subcutaneous single doses of rHu-EPO.

THEORETICAL

Dynamics of the Reticulocyte and Mature RBC System

The reticulocyte count, RET, is determined by the balance between the production rate, $k_R(t)$, and elimination rate, $k_{out}(t)$:

$$\frac{dRET}{dt} = k_R(t) - k_{out}(t) \tag{1}$$

If one assumes that the reticulocytes have known lifespan distributions that can change with time due to the drug treatment, then the reticulocyte lifespan distribution uniquely defines $k_{out}(t)$. As shown in (12), $k_{out}(t)$ can be calculated by the convolution of $k_R(t)$ with the lifespan (τ) distribution probability density function (p.d.f.) $\ell(t,\tau)$:

$$k_{out}(t) = \int_0^\infty k_R(t-\tau)\ell(t-\tau,\tau)d\tau \tag{2}$$

If each reticulocyte becomes a mature erythrocyte after its lifespan expires, then $k_{out}(t)$ is the production rate for the mature RBC (MRBC). We assume that each mature RBC has a fixed lifespan, T_{RBC}, which in adult healthy humans is about 120 days. Then one can write the equation describing the change of MRBC in time (11):

$$\frac{dMRBC}{dt} = k_{out}(t) - k_{out}(t - T_{RBC}) \tag{3}$$

Figure 1 illustrates the RET-MRBC system. Prior to drug treatment the reticulocytes were at steady-state with the

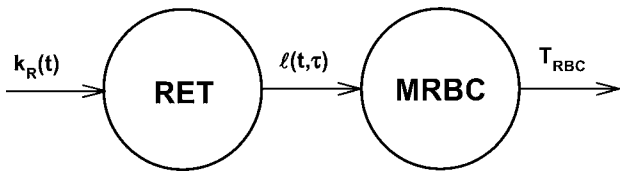

Fig. 1. Schematic diagram of the model describing the turnover of reticulocytes and RBC in blood. Reticulocytes are released from the bone marrow to blood at an arbitrary, time dependent rate $k_R(t)$. The conversion rate of reticulocytes to mature RBC is determined by the reticulocyte lifespan distribution that can depend on time $\ell(t,\tau)$. Mature RBC are eliminated from blood due to senescence after time T_{RBC}. RBC are calculated as the sum of reticulocytes and mature RBC.

constant production rate, k_{R0}, and time independent lifespan distribution $\ell_0(\tau)$ related by the baseline (12):

$$\mathrm{RET}_0 = k_{R0} \cdot T_{RET0} \tag{4}$$

where T_{RET0} is the baseline mean reticulocyte lifespan. A similar relationship holds for MRBC (11):

$$\mathrm{MRBC}_0 = k_{R0} \cdot T_{RBC} \tag{5}$$

If the drug was administered at time $t=0$, then for times $t < T_{RBC}$ the MRBC elimination rate is constant:

$$k_{out}(t - T_{RBC}) = k_{R0} \tag{6}$$

As the RBC count in blood is the sum of RET and MRBC, then adding side-by-side Eqs. 1 and 3, an equation describing the change of RBC in time can be obtained:

$$\frac{\mathrm{dRBC}}{\mathrm{d}t} = k_R(t) - k_{out}(t - T_{RBC}) \tag{7}$$

which after integration yields for times $t < T_{RBC}$ the following relationship:

$$\mathrm{RBC}(t) = \mathrm{RBC}_0 + \int_0^t \Delta k_R(z)\mathrm{d}z \tag{8}$$

where $\Delta k_R(t)$ denotes the change of $k_R(t)$ from the baseline value:

$$\Delta k_R(t) = k_R(t) - k_{R0} \tag{9}$$

Equation 9 states that theoretically the RBC data alone suffices to determine the reticulocyte production rate $k_R(t)$.

The Precursor–Successor Relationship Between Reticulocyte and RBC

Assuming that the reticulocyte lifespan distribution does not depend on time and each reticulocyte has the same lifespan T_{RET0}, then the lifespan distribution is centered at T_{RET0} and the p.d.f. $\ell(t,\tau)$ becomes the

$$\ell(t,\tau) = \delta(\tau - T_{RET0}) \tag{10}$$

where $\delta(\tau)$ denotes the Dirac delta function. In this case Eq. 1 can be integrated yielding the following relationship between RET and k_R (11):

$$\mathrm{RET}(t) = \int_{t-T_{RET0}}^{t} k_R(z)\mathrm{d}z \tag{11}$$

Equation 11 can be used to express the integral in Eq. 8 and derive an equation for RBC(t) in terms of RET(t) evaluated at finite number of times t delayed by multiples of T_{RET0} (see Appendix A)

$$\Delta\mathrm{RBC}(t) = \sum_{i=0}^{N} \Delta\mathrm{RET}(t - i \cdot T_{RET0}) \text{ for } t < T_{RBC} \tag{12}$$

where $N = \mathrm{INT}(t/T_{RET0})$ is the integer describing how many times t is bigger than T_{RET0} and ΔRBC(t) and ΔRET(t) denote the changes from the baselines at time t:

$$\begin{aligned} \Delta\mathrm{RBC}(t) &= \mathrm{RBC}(t) - \mathrm{RBC}_0 \text{ and} \\ \Delta\mathrm{RET}(t) &= \mathrm{RET}(t) - \mathrm{RET}_0 \end{aligned} \tag{13}$$

Briefly, if fixed life spans for RET and MRBC are assumed, then the change in the RBC count at time t is the sum of the changes in the reticulocyte counts occurring at times t, $t - T_{RET0}$, ..., up to $t - (N+1)T_{RET0}$. Equation 12 constitutes the precursor–successor relationship between RET and RBC since at any moment of time, the RBC count can be determined solely from the reticulocyte counts at specific times. Figure 2 shows a comparison of the observed RBC count in peripheral blood with RBC predicted by Eq. 12 in an adult healthy human.

rHu-EPO Effect on the Reticulocyte Lifespan Distribution

As seen in Fig. 2 the assumption of fixed life spans for RET and RBC is not valid for the RET-MRBC system stimulated by rHu-EPO. The observed discrepancy between ob-

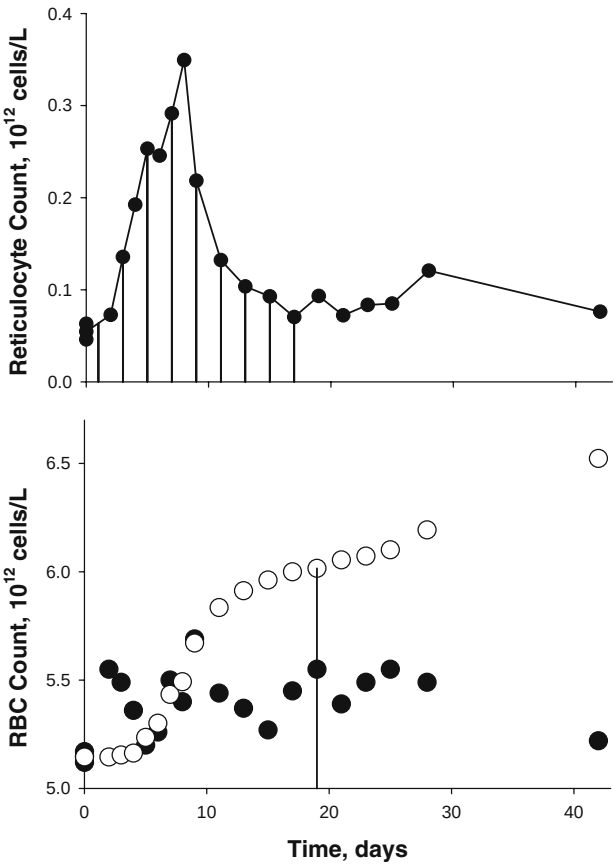

Fig. 2. Reticulocyte and RBC counts for an individual patient (*solid symbols*). The RBC count predicted by the precursor–successor relationship is represented by open symbols. According to this relationship, the change from the baseline in RBC at time t can be determined by summing up the changes from the baseline of the reticulocyte counts at times $t - T_{RET0}$, $t - 2T_{RET0}$, etc. up to N times.

served and predicted RBC values can be removed by assuming a transient effect of rHu-EPO treatment on lifespan distribution that can be described by an empirical p.d.f.:

$$\ell(t,\tau) = (1-\chi(t))\delta(\tau - T_{\mathrm{RET}0}) + \chi(t)\delta(\tau - T_{\mathrm{RET}}) \qquad (14)$$

where $\chi(t)$ is a jump function between the times T_0 and T_1

$$\chi(t) = \begin{cases} 1, \text{ if } T_0 \le t \le T_1 \\ 0, \text{ if } t < 0 \text{ or } T_1 < t \end{cases} \qquad (15)$$

assuming that the drug was administered at time $t=0$. The function $\chi(t)$ represents the effect of drug action on the reticulocyte lifespan distribution. According to Eqs. 14 and 15 the drug switches the distribution of life spans to the point distribution centered at T_{RET} for times in the interval $T_0 \le t \le T_1$, otherwise the lifespan distribution is centered at the baseline lifespan $T_{\mathrm{RET}0}$ which we assume is less than T_{RET}. The parameter T_0 is interpreted as the lag time between rHu-EPO administration and its effect on the lifespan distribution. The difference $\Delta T_1 = T_1 - T_0$ can be interpreted as the duration of drug effect on the lifespan distribution. In the case $T_{\mathrm{RET}0} = T_{\mathrm{RET}}$, the p.d.f. $\ell(t,\tau)$ reduces to the point distribution described by Eq. 10. The convolution integral in Eq. 2 that defines $k_{\mathrm{out}}(t)$ now can be explicitly calculated:

$$k_{\mathrm{out}}(t) = k_{\mathrm{R}}(t - T_{\mathrm{RET}0})(1 - \chi(t - T_{\mathrm{RET}0}))(+k_{\mathrm{R}}(t - T_{\mathrm{RET}})\chi(t - T_{\mathrm{RET}}) \qquad (16)$$

Similarly to Eq. 12, this form of the reticulocyte conversion rate allows the integration of Eq. 1 and obtains the following relationship (see Appendix B):

$$\mathrm{RET}(t) = \int_{t-T_{\mathrm{RET}0}}^{t} k_{\mathrm{R}}(z)\mathrm{d}z + \int_{t-T_{\mathrm{RET}}}^{t-T_{\mathrm{RET}0}} k_{\mathrm{R}}(z)\chi(z)\mathrm{d}z \qquad (17)$$

The second integral in Eq. 17 describes the change in reticulocyte counts caused by the transient rHu-EPO effect on the reticulocyte lifespan distribution.

RHu-EPO Effect on the Reticulocyte Production Rate

Theoretically, the RBC data suffices to recover the $k_{\mathrm{R}}(t)$ function via Eq. 8 and evaluate the effect of the drug treatment on reticulocyte production. However, because of data variability such information is of limited value. The simplistic form of the function $\chi(t)$ allows one to use the reticulocyte data for this purpose, which is less variable. Although deconvolution techniques are available to obtain the $k_{\mathrm{R}}(t)$ profile (13), such results might be difficult to interpret. Instead we postulate an empirical function describing $k_{\mathrm{R}}(t)$ by means of a few parameters that have clear physiological interpretations. Figure 3 shows that except for the lag time in reticulocyte response to rHu-EPO and extended duration of the stimulatory effect, one can observe a rebound. These three processes were accounted for in the following step function

$$k_{\mathrm{R}}(t) = \begin{cases} k_{\mathrm{R}0}, & t \le T_0 \\ k_{\mathrm{R}1}, & T_0 < t \le T_1 \\ k_{\mathrm{R}2}, & T_1 < t \le T_2 \\ k_{\mathrm{R}0}, & t > T_2 \end{cases} \qquad (18)$$

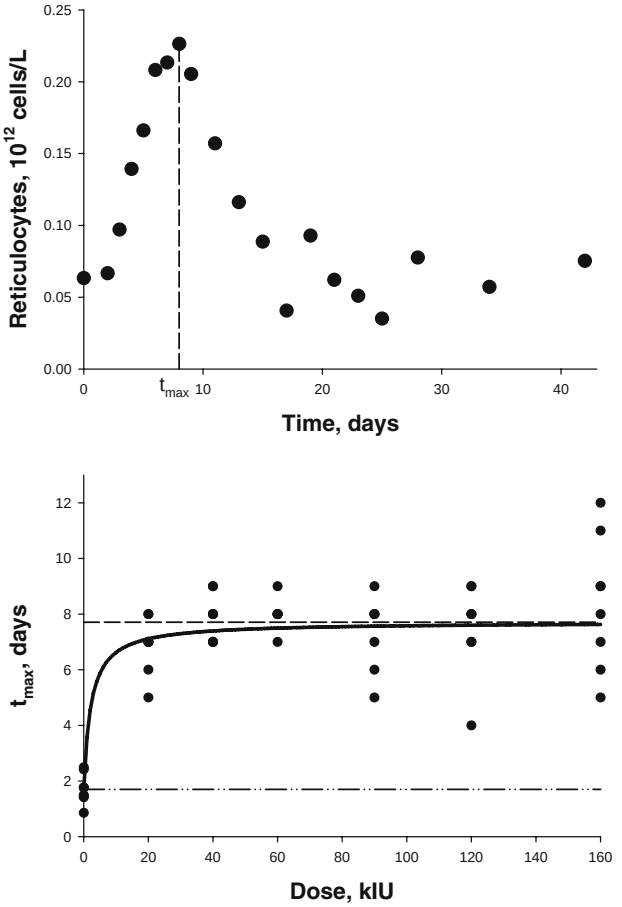

Fig. 3. The time of maximum reticulocyte count ($t_{\max}$) for a representative subject (*upper panel*) and the plot of $t_{\max}$ for all subjects vs. rHu-EPO dose. For the placebo group $t_{\max} = T_{\mathrm{RET}0}$ that was calculated from the baseline equation. The *solid line* indicates the fit of the $E_{\max}$ equation with the estimated parameters $E_{\max} = 7.7$ days (*dashed line*), $E_0 = 1.7$ days (*dotted dashed line*), and $\mathrm{ED}_{50} = 2.2$ kIU.

where we assume that $k_{\mathrm{R}2} \le k_{\mathrm{R}0} \le k_{\mathrm{R}1}$. The profile of the function $k_{\mathrm{R}}(t)$ is shown in Fig. 4. The rebound in the reticulocyte production occurs between the times T_1 and T_2. The simplistic forms of functions $\chi(t)$ and $k_{\mathrm{R}}(t)$ allows the integration of the equations for RET(t) and RBC(t) (see Appendix B). If $T_{\mathrm{RET}0} < T_{\mathrm{TRET}}$, $T_0 + T_{\mathrm{RET}} < T_1$, and $T_1 + T_{\mathrm{RET}} < T_2$, then

$$\mathrm{RET}(t) = \begin{cases} \mathrm{RET}_0, & t \le T_0 \\ \Delta k_{\mathrm{R}1}(t - T_0) + \mathrm{RET}_0, & T_0 \le t \le T_0 + T_{\mathrm{RET}0} \\ k_{\mathrm{R}1}(t - T_0), & T_0 + T_{\mathrm{RET}0} \le t \le T_0 + T_{\mathrm{RET}} \\ k_{\mathrm{R}1}T_{\mathrm{RET}}, & T_0 + T_{\mathrm{RET}} \le t \le T_1 \\ -(k_{\mathrm{R}1} - k_{\mathrm{R}2})t + k_{\mathrm{R}1}(T_1 + T_{\mathrm{RET}}) - k_{\mathrm{R}2}T_1, & T_1 \le t \le T_1 + T_{\mathrm{RET}0} \\ -k_{\mathrm{R}1}t + k_{\mathrm{R}1}(T_1 + T_{\mathrm{RET}}) + k_{\mathrm{R}2}T_{\mathrm{RET}0}, & T_1 + T_{\mathrm{RET}0} \le t \le T_1 + T_{\mathrm{RET}0} + \Delta T \\ k_{\mathrm{R}2}T_{\mathrm{RET}0}, & T_1 + T_{\mathrm{RET}0} + \Delta T \le t \le T_2 \\ \Delta k_{\mathrm{R}2}t - \Delta k_{\mathrm{R}2}T_2 + k_{\mathrm{R}2}T_{\mathrm{RET}0}, & T_2 \le t \le T_2 + T_{\mathrm{RET}0} \\ \mathrm{RET}_0, & T_2 + T_{\mathrm{RET}0} \le t \end{cases} \qquad (19)$$

and

$$\mathrm{RBC}(t) = \begin{cases} \mathrm{RBC}_0, & t \le T_0 \\ \mathrm{RBC}_0 + \Delta k_{\mathrm{R}1}(t - T_0), & T_0 \le t \le T_1 \\ \mathrm{RBC}_0 - \Delta k_{\mathrm{R}2}(t - T_1) + \Delta k_{\mathrm{R}1}\Delta T_1, & T_1 \le t \le T_2 \\ \mathrm{RBC}_0 - \Delta k_{\mathrm{R}2}\Delta T_2 + \Delta k_{\mathrm{R}1}\Delta T_1, & T_2 \le t \le T_{\mathrm{RBC}} \end{cases} \qquad (20)$$

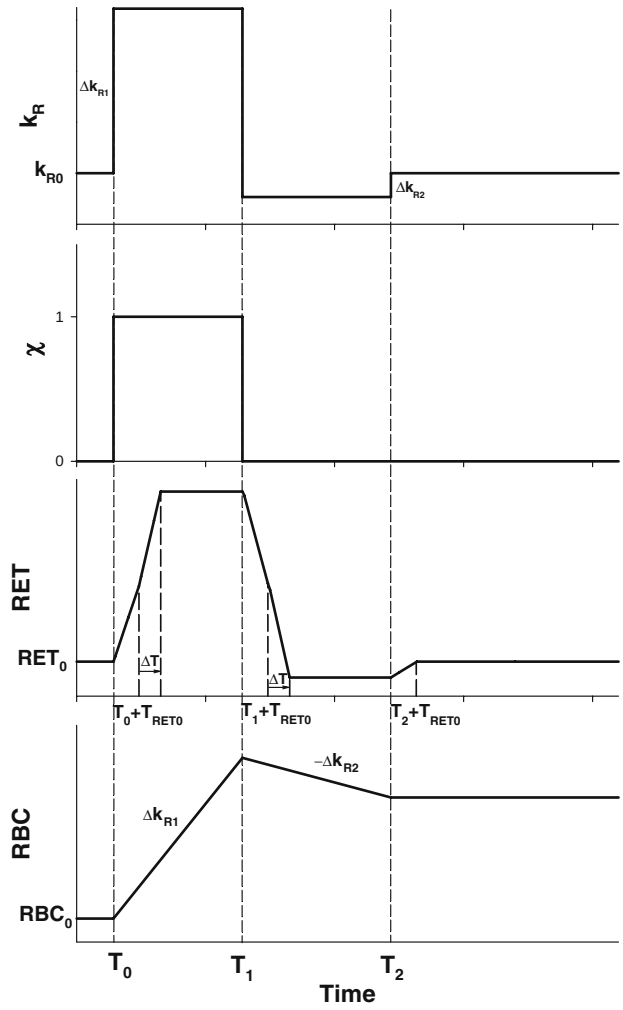

Fig. 4. Empirical time profiles of the reticulocyte production rate $k_R(t)$ and the time dependent factor $\chi(t)$ in the reticulocyte lifespan distribution following a single dose administration of rHu-EPO augmented by the subsequent time courses of reticulocyte and RBC counts as predicted by the model. The effect of rHu-EPO on $k_R(t)$ was described by simplistic step functions for the lag time T_0, duration of the stimulatory effect $\Delta T_1 = T_1 - T_0$ and the change from the baseline $\Delta k_{R1} = k_{R1} - k_{R0}$, as well as duration of the rebound effect $\Delta T_2 = T_2 - T_1$ and the change from the baseline $\Delta k_{R2} = k_{R0} - k_{R2}$. The rHu-EPO effect on the reticulocyte lifespan distribution was assumed to coincide with the stimulatory effect and was modeled by the jump function of value 1 and duration $\Delta T_1 = T_1 - T_0$. The durations of the onset and offset parts of the reticulocyte response are determined by the increased reticulocyte lifespan $\Delta T = T_{RET} - T_{RET0}$. At the times $T_0 + T_{RET0}$ and $T_1 + T_{RET0}$ one can observe kinks in the reticulocyte response curve caused by a temporary change in the reticulocyte lifespan distribution. The period of the maximum reticulocyte count is $T_1 - T_{RET}$ and the rebound minimum lasts from $T_1 + T_{RET}$ to T_2. The reticulocyte count returns to the baseline at time $T_2 + T_{RET0}$. The RBC counts increases at the rate Δk_{R1} to the peak value attained at time T_1. The rebound effect causes RBC to decline at the rate Δk_{R2} to plateau at time T_2. All profiles are valid for times no greater than T_{RBC}.

where $\Delta k_{R1} = k_{R1} - k_{R0}, \Delta k_{R2} = k_{R0} - k_{R2}, \Delta T_2 = T_2 - T_1$, and $\Delta T = T_{RET} - T_{RET0}$. The parameter Δk_{R1} characterizes the stimulatory effect of rHu-EPO on the reticulocyte production, whereas Δk_{R2} describes the depth of the rebound effect of duration ΔT_2. Equation 20 holds true without the conditions under which Eq. 19 was derived. As mentioned above, all parameters describing $k_R(t)$ should be theoretically identifiable from the RBC data, however the reticulocyte counts also provide information necessary to determine the parameter values although in a more complex form.

MATERIALS AND METHODS

Data

RHu-EPO plasma concentrations as well as hematological responses were obtained from an open-label, randomized, parallel-design, single-center study (14). Fifty-six healthy male subjects were randomly assigned in equal numbers ($n = 8$ per group) to 7 study treatment groups. The study treatment groups received single fixed s.c. doses of epoetin alfa at levels 20, 40, 60, 90, 120, and 160 kIU. A control group was also included in the study, and the subjects in this group did not receive a drug injection but underwent all other study procedures. Blood collection for determination of pharmacokinetic and pharmacodynamic data began prior to drug administration on study Day 1 and continued at scheduled time points through study Day 43. Total study duration was 43 days. Blood samples (2.5 ml) for determination of erythropoietin serum concentration were collected at 30, 20, and 10 min before drug administration and at 0.5, 1, 2, 5, 8, 12, 18, 24, 30, 36, 48, 60, 72 h after administration. For erythropoietin pharmacodynamic measurements blood samples were collected 30 and 10 min prior to drug administration on day 1, daily for days 2–10, every other day for days 12–26, and on days 29, 35, and 43. Additional blood samples were collected for iron measurements, serum chemistry, and genetic analysis. The total volume of blood collected within the first day of study did not exceed 40 ml. Approximately 120 ml of blood, in total, was colleted for pharmacokinetic blood samples. The total quantity of blood drawn from each subject who completed the study was about 350 ml. The study was conducted in accordance with principles for human experimentation as defined in the *Declaration of Helsinki* and was approved by the Human Investigational Review Board of the study center. Informed consent was obtained from each subject after being told the potential risks and benefits, as well as the investigational nature of the study.

Precursor–Successor Relationship

For each dose and each patient the RET_0 and RBC_0 were calculated as the mean of three points of pretreatment data. For the control group, all data were used to calculate RET_0 and RBC_0. The baseline reticulocyte lifespan values, T_{RET0}, were subsequently obtained from Eqs. 4 and 5 as follows

$$T_{RET0} = \frac{RET_0}{MRBC_0} T_{RBC} \tag{21}$$

where T_{RBC} 120 days. Equation 12 was used to determine RBC count at time t. To calculate $RET(t - i \cdot T_{RET0})$ the reticulocyte data were linearly interpolated as shown in Fig. 2 (upper panel). The interpolation procedure and the precursor–successor

relationship were implemented in NONMEM (15) to obtain the predicted RBC values at observed times. A NONMEM control stream containing this procedure and a representative data file are shown in Appendix C.

Reticulocyte Peak Time Dependence on Dose

The reticulocyte peak times, t_{max}, for all subjects receiving rHu-EPO were determined from the data. The t_{max} values for the control group were equal to T_{RET0}. The relationship between the t_{max} and rHu-EPO dose, D, administered to a subject was described by the E_{max} equation:

$$E = E_0 + \frac{(E_{max} - E_0) \cdot D}{ED_{50} + D} \tag{22}$$

where E_0 denoted the model predicted T_{RET0}, and E_{max} the maximal value of the reticulocyte peak time. Equation 22 was fitted to the pooled t_{max} data as a fixed effect model in NONMEM (15) with dose as the independent variable. The residual random effect model consisted of the additive error model describing the residual variability

$$t_{max} = E + \boldsymbol{\varepsilon} \tag{23}$$

where t_{max} is the observed reticulocyte peak times, E is the corresponding model predicted reticulocyte peak times, and ε is an independent normally distributed random variables with zero mean and variance, σ^2.

Resolution of Parameters Describing k_R and $\ell(t,\tau)$

Although explicit solutions for RET and RBC were derived (see Eqs. 19 and 20), their implementation into a computer program would require consideration of a large number of cases dictated by unknown relationships between parameters T_{RET0}, T_{RET} and T_0, T_1, T_2. Instead the integral representations of RET and RBC in Eqs. 8 and 17 were used based on the explicit solution of the integral

$$\int_0^x k_R(z)dz = \begin{cases} k_{R0}x, & x \le T_0 \\ k_{R1}(x - T_0) + k_{R0}T_0, & T_0 \le x \le T_1 \\ k_{R2}(x - T_1) + k_{R1}(T_1 - T_0) + k_{R0}T_0, & T_1 \le t \le T_2 \\ k_{R0}(t - T_2) + k_{R2}(T_2 - T_1) + k_{R1}(T_1 - T_0) + k_{R0}T_0, & t \ge T_2 \end{cases} \tag{24}$$

where $x = t$, $t - T_{RET0}$. A similar relationship was used to calculate the integral $\int_0^x k_R(z)\chi(z)dz$. The integrals present in Eqs. 8 and 17 were expressed as the differences between the integrals calculated in Eq. 24 with the appropriate x values. Equations 8 and 17 determined the fixed effect models describing the reticulocyte and RBC count data for each subject receiving rHu-EPO using NONMEM (15). The additive error model was used to describe the residual variability. The estimated parameters were T_0, ΔT_1, ΔT, Δk_{R1}, Δk_{R2}, and the variance of the residual variability, σ^2. A NONMEM code and a fragment of a data set for a representative subject are shown in Appendix D.

Dose Dependence of Parameters Describing k_R and $\ell(t,\tau)$

The estimates of the fixed model parameters for each subject were pooled and analyzed as functions of rHu-EPO dose. The E_{max} equation Eq. 22 was used to fit separately the individual estimates of ΔT_1, ΔT, Δk_{R1}, Δk_{R2}, and T_0. Similarly, the estimations were performed by NONMEM using the additive residual error model and first order conditional estimation (FOCE) method (15). To test if the dose dependence is significant the 95% confidence intervals for the E_{max} estimate were calculated using non-parametric bootstrap (16) implemented in the package Wings for NONMEM (N. Holford, Version 4.04, June 2003, Auckland, New Zealand). For the non-parametric bootstrap, a new replication of the original dataset (a bootstrap sample) was obtained by random draws of individual data (with replacement) from the original dataset. The model was re-fitted to each new dataset and this process was repeated 1,000 times with different random draws. Bootstrap runs with unsuccessful minimization were excluded from further analysis. The 95% confidence intervals for the model parameters were calculated as the 2.5th and 97.5th percentiles of the parameter estimates obtained from each non-parametric bootstrap replicates. If the estimated 95% confidence intervals for the E_{max} value included the E_0 value, then the dose dependence was not present, otherwise dose dependence was concluded.

RESULTS

One subject from the 60 and 90 kIU dosing groups was not included in our data set for analysis because of an

Table I. The Mean (±Standard Deviation) Values of the Baseline Reticulocyte (RET_0) and RBC (RBC_0) Counts, Reticulocyte Life Spans (T_{RET0}), Reticulocyte Production Rates (k_{R0}) and Range of Probabilities (P) for the Two-sided t Test on the Difference Between the Mean Values of Observed RBC and Predicted by Eq. 12

Dose kIU	RET_0 10^{12} cells/l	RBC_0 10^{12} cells/l	T_{RET0} days	k_{R0} 10^9 cells/l/day	P
0	0.07 ± 0.02	4.96 ± 0.4	1.71 ± 0.5	50.1 ± 8.9	0.14–0.98
20	0.07 ± 0.03	4.91 ± 0.3	1.81 ± 0.7	40.3 ± 2.5	0.051–0.97
40	0.06 ± 0.02	4.97 ± 0.2	1.53 ± 0.4	40.9 ± 2.1	0.005–0.82
60	0.06 ± 0.01	5.10 ± 0.2	1.43 ± 0.2	42.0 ± 1.2	0–0.95
90	0.08 ± 0.03	4.79 ± 0.3	1.92 ± 0.8	39.3 ± 2.5	0.010–0.88
120	0.06 ± 0.02	4.61 ± 0.2	1.63 ± 0.6	37.9 ± 2.0	0–0.89
160	0.09 ± 0.04	4.91 ± 0.2	2.10 ± 1.0	40.2 ± 2.0	0.024–0.84

RET_0 and RBC_0 were Obtained from Each Subject from the Pretreatment Data, T_{RET0} was Estimated by Fitting Eq. 12 to Individual RBC Counts and k_{R0} was Calculated from Eq. 4

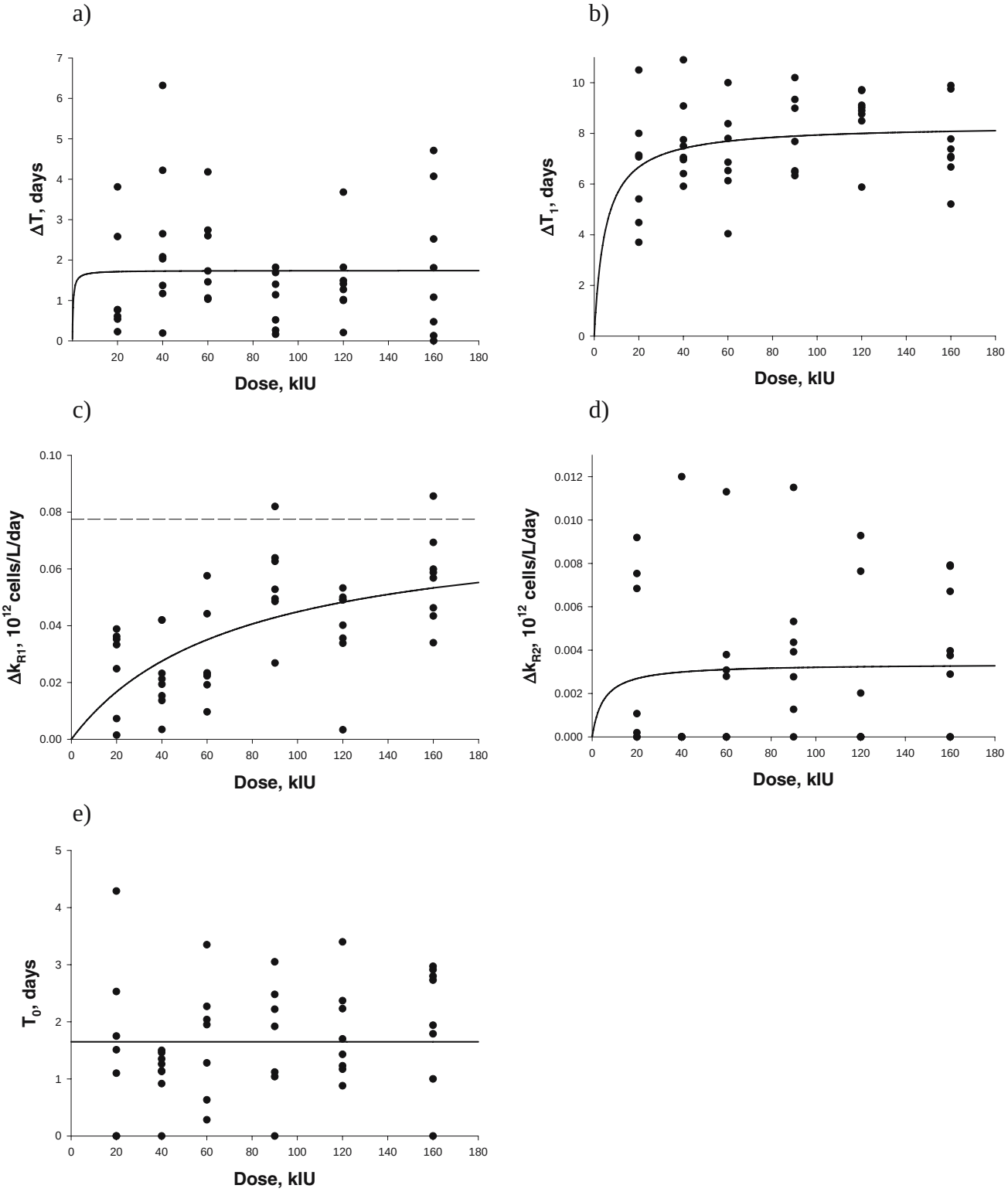

Fig. 5. Observed and model predicted effects of rHu-EPO on the individual parameter estimates as a function of dose: (a) change in the reticulocyte lifespan, ΔT, (b) duration of rHu-EPO stimulatory effect, ΔT_1, (c) change from the baseline of the reticulocyte production rate due to the rHu-EPO stimulatory effect, Δk_{R1}, (d) change from the baseline of the reticulocyte production rate due to the rHu-EPO rebound effect, Δk_{R2}, (e) the lag time in reticulocyte response, T_0.

incomplete reticulocyte profile that was terminated before reaching the peak value. One subject from 20 kIU dosing group was also excluded because of the abnormal RBC time course.

Prediction of RBC Counts from the Precursor–Successor Relationship

If fixed life spans for reticulocytes and RBC are assumed, then T_{RET0} for each subject can be calculated from the baseline counts. Subsequently, the precursor–successor relationship between RET and MRBC can be established, and an explicit equation predicting RBC counts solely from the reticulocyte counts was derived. Equation 12 was used to calculate RBC at observation times and compare the predictions with the observed values. The predicted RBC time courses exhibited an initial 3–4 day lag time, rapid onset followed by a plateau that was reached about day 13. For all doses the mean predictions were greater than mean observations except for a few initial points. Figure 2 shows an RBC vs. time profiles for a representative subject. To test if the predicted RBC was significantly different from observed RBC, we assumed that at each time point the predictions and observations were normally distributed. Since the predictions were correlated, only the probabilities that prediction was different than observation were calculated at separate time points. The range of such probabilities was recorded in Table I. The mean predictions were significantly higher than mean observations for dose groups 60, 90, 120, and 160 kIU. The lowest probability was for the 60 KIU dose group. For these doses, the null hypothesis that the observations are the same as predictions can be rejected. Consequently, either the assumption about the fixed life spans is violated or some fraction of reticulocytes did not mature to RBC violating the precursor–successor relationship. We explored the former possibility assuming that there was no loss of reticulocytes during their conversion to RBC.

T_{max} Dependence on Dose

If the reticulocyte lifespan was fixed and the reticulocyte production rate $k_R(t)$ had a rapid onset to a peak value followed by a gradual return to the baseline, then according to the argument presented in (11), the difference between the peak time t_{max} and the lag time in reticulocyte responses should coincide with the reticulocyte lifespan T_{RET0}. The t_{max} values were obtained for all treated subjects and plotted against rHu-EPO dose as shown in Fig. 3. Fitting of the E_{max} equation resulted in the estimate of $E_{max} = 8.45$ days with the 95% confidence interval (95%CI) 7.59 to 9.39. Since the estimated value E_0 of the baseline reticulocyte lifespan was 1.65 (95%CI: 1.38–1.94) days we concluded that t_{max} is depended on dose. Consequently, $k_R(t)$ might have a different profile than postulated above or the reticulocyte lifespan could change after rHu-EPO treatment.

Characterization of Time Courses of $k_R(t)$ and $\ell(t,\tau)$

The resolution of the $k_R(t)$ from the reticulocyte data cannot be obtained without knowledge of the reticulocyte conversion rate $k_{out}(t)$. Since the results presented above indicated that rHu-EPO affects the lifespan distribution, we postulated the simplest empirical function that would account for temporal changes described by Eqs. 14 and 15. After a lag time, the rHu-EPO treatment shifts T_0, the point reticulocyte lifespan distribution centered at T_{RET0}, to the point distribution centered at T_{RET}. The duration of this change is $\Delta T_1 = b\,T_1 - T_0$ after which the distribution is centered again at T_{RET0}. Such an approach allowed us to introduce a parameter characterizing the duration of rHu-EPO effect on the reticulocyte lifespan. To be able to determine the values of T_0, ΔT, and ΔT_1 we also assumed that the duration of the rHu-EPO effect on $k_R(t)$ is ΔT_1 and can be characterized by a single parameter $\Delta k_{R1} = k_{R1} - k_{R0}$ representing the increase of the reticulocyte production from the baseline value. The observed rebound in the reticulocyte data was modeled as a decrease in $k_R(t)$ of duration $\Delta T_2 = T_2 - T_1$ and value $\Delta k_{R2} = k_{R0} - k_{R2}$ immediately following the increase. However, for the majority of the subjects ΔT_2 was not able to be estimated with an acceptable precision and was fixed at 42 days, the last data time point for all subjects. This forced the rebound to occur beyond the last measurable data point if $\Delta k_{R2} > 0$, and only the apparent rebound intensity was estimated.

The simultaneous fittings of Eqs. 8 and 17 to the individual subject RBC and reticulocyte counts resulted in estimates of T_0, ΔT, ΔT_1, Δk_{R1}, and Δk_{R2} (see Fig. 5). To test if these parameters depend on dose, the E_{max} model presented in Eq. 22 was fitted to the pooled parameter values, and significant difference between E_{max} and E_0 was claimed if the 95% confidence interval for the E_{max} estimate did not include the E_0 value. As shown in Table II, only the E_{max} value for the T_0 individual estimates was not different

Table II. Parameter Estimate Values (95% Confidence Interval) of the E_{max} Model Fitted to the Pharmacodynamic Characteristics of all Subjects as a Function of Dose

Parameter	T_0*, days	ΔT_1, days	Δk_{R1}, 10^9 cells/l/day	Δk_{R2}, 10^9 cells/l/day	ΔT, days
E_0	1.65 (1.38–1.94)	–	–	–	–
E_{max}	–	8.33 (7.51–9.26)	77.5 (49.9–97.6)	3.73 (2.20–9.10)	1.74 (1.37–2.55)
ED_{50}, kIU	–	4.98 (0–14.10)	72.7 (21.35–122)	5.07 (0–147)	0.33 (0–22.70)
σ	0.99 (0.80–1.19)	1.65 (1.30–1.91)	16.6 (13.0–19.8)	3.71 (2.83–4.30)	1.40 (1.00–1.71)

*The values of T_0 where fitted by the constant equation $E = E_0$.

from E_0, and therefore the lag time in the rHu-EPO effect on $k_R(t)$ and $\ell(t,\tau)$ was found to be dose independent, with a typical value of 1.65 days. The remaining parameters, exhibited dependence on dose, as the E_{max} estimates were different than 0, which was the fixed value for E_0. The estimated maximum value of ΔT was 1.74 days indicating that rHu-EPO transiently increases the reticulocyte lifespan from its mean baseline value $T_{RET0} = 1.74$ days to $T_{RET} = 3.39$ days. The estimated value of the duration of this effect was 8.3 days. The low value of ED50 = 0.3 kIU for the ΔT estimates implies that rHu-EPO administration changes the reticulocyte lifespan even for low doses. According to our assumption, the same duration of effect applies to the increase of $k_R(t)$ with the estimated value of $ED_{50} = 4.98$ kIU. The maximum estimated value of Δk_{R1} was $77 \cdot 10^9$ cells/l/day which translates into 88% increase with respect to the mean baseline of $k_{R0} = 41 \cdot 10^9$ cells/l/day. The estimated value of ED_{50} for this parameter was 72 kIU, yielding 36% and 53% of the maximum effect predicted for the dose of 40 and 80 kIU, respectively. Finally, the estimated maximum effect of the rebound phenomena was $3.7 \cdot 10^9$ cells/l/day, which was 9% of the baseline production. Similarly to ΔT_1, the estimated ED_{50} value for Δk_{R2} was 5.07 kIU.

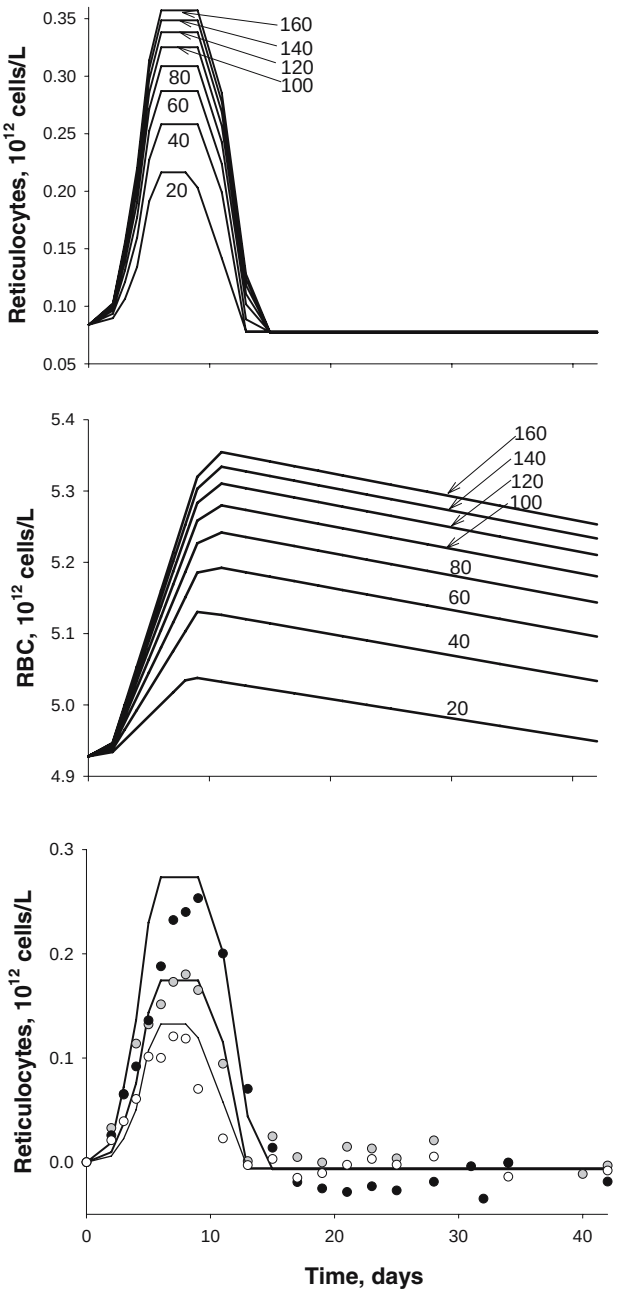

Fig. 6. Simulated reticulocyte and RBC responses following single s.c. dose of rHu-EPO. The parameters T_0, ΔT, ΔT_1, Δk_{R1}, and Δk_{R2} were calculated from the E_{max} equations describing their dependence on dose using the estimated values presented in Table II. Reticulocytes and RBC were simulated according to Eqs. 8 and 17. The indicated doses are expressed in kIU. The lower panel shows the simulated reticulocyte counts superimposed on the observed reticulocyte counts corrected by the baseline values for doses 20, 40, and 160 kIU. The symbols denote the mean values for each dosing group.

Simulations of RET and RBC Time courses

The E_{max} equations describing the relationships between parameters T_0, ΔT, ΔT_1, Δk_{R1}, and Δk_{R2}, and dose were used to determine their values for an array of doses. Equations 8 and 17 were used to simulate RET and RBC time profiles shown in Fig. 6. The baseline values RET_0 and RBC_0 were obtained as the mean of the placebo group combined with the pre-dose measurements from the treatment groups. Both RET and RBC counts increased with dose, but the differences between these responses became smaller. For example the peak RBC count increase from the baseline caused by a dose of 20 kIU was 0.11×10^{12} cells/l. Compared to the 20 kIU dose, the 40 kIU dose yielded an 84% increase in the peak of the RBC response corrected by the baseline, which was further increased by 54% when the dose increased to 80 kIU. Since the ED_{50} value for Δk_{R2} was 5 kIU, all RET responses exhibited the maximal rebound of 8% of the baseline, which was observed as a negative slope in the RBC responses.

DISCUSSION

The assumption of a fixed lifespan for all reticulocytes made in our previous models of the stimulatory effect of rHu-EPO on the reticulocyte and RBC (4,17,18) was dictated by the rule of parsimony and yielded satisfactory results. However, the obtained estimates of T_{RET0} in the range 3–7 days (4,11,18), made the predictions of T_{RBC} based on the baseline relationship much larger than 120 days. This flaw could not be removed by considering in our PD models arbitrary reticulocyte lifespan distributions described by the gamma and log-normal functions (12). The over-prediction of the RBC counts by the precursor–successor relationship and the dose dependence of t_{max} indicated that administration of rHu-EPO affects the reticulocyte lifespan distribution. Our simplistic and empirical way of assessing this effect yielded an increase by 1.74 days for all considered doses. The maximal duration of the change in the lifespan was about 8 days.

Our approach towards empirical modeling of the reticulocyte production rate using simplistic step functions is based on the work of Uehlinger *et al.* (5) where the hematocrit time profiles in anemic patients were integrated step-like RBC survivor functions. This technique has been elaborated using a linear system analysis where the reticulocyte count was described as the convolution of the reticulocyte production rate with the unit impulse response UIR(t) (19). The production rate was a function of erythropoietin serum concentration whereas UIR(t) was a jump function with a lag time and duration of the response equal to the fixed reticulocyte lifespan. This model has been extended further to describe reticulocyte, RBC, and hemoglobin responses to endogenous erythropoetin in phlebotomized sheep (20). In our approach we purposely omitted rHu-EPO serum concentration control of the reticulocyte production and derived the integral relationships from the compartmental indirect response models rather than a system-structure independent convolution type model. The p.d.f. of reticulocyte lifespan distribution $\ell(t,\tau)$ plays a role similar to the UIR(t), although qualitatively these two functions are different. The relationships presented here can be formulated using the linear system convolution type formalism, including the relationship between reticulocytes and the reticulocyte production rate (13).

The step-like function describing $k_R(t)$ did not depend on rHu-EPO serum concentrations $C_{EPO}(t)$. Including rHu-EPO into the model would require additional assumptions about functional relationship between $C_{EPO}(t)$ and $k_R(t)$ and, consequently lead to a limited generality of our findings about the time profile of $k_R(t)$. This would also prohibit testing the dose dependence of the components of $k_R(t)$ such as T_0, ΔT_1, and Δk_{R1}. Instead, the information gained from our study about the shape of the $k_R(t)$ function can be further utilized in a development of a more mechanistic pharmacokinetic-pharmacodynamic model that will take into account both $k_R(t)$ dependence on $C_{EPO}(t)$ and the rHu-EPO effect on the reticulocyte lifespan distribution. The proposed shape of the reticulocyte production rate $k_R(t)$ limits applications of our model to data comprising of reticulocyte and RBC responses to a single dose of rHu-EPO. For other dosing regimens a more relevant functional form should be proposed.

Increases of t_{max} and ΔT_1 with dose indicates a rHu-EPO effect on reticulocyte lifespan. An increase of the maturation time of the reticulocyte population in the circulation after administration of a single dose of rHu-EPO to healthy volunteers has been reported previously (7). This is may be caused by an earlier release of immature reticulocyte from bone marrow to blood as well as increased number of younger reticulocytes produced from the bone marrow progenitor cells stimulated by rHu-EPO (7). A shift in the circulating reticulocytes age distribution from older to younger cells may lead to a transient increase in their lifespan. The hematological markers of reticulocyte age HFR, MFR and LFR have been reported to reflect such a behavior in time in phlebotomy-induced stress erythropoiesis (8).

Although maturation to erythrocytes is the major process for depleting the reticulocyte pool in plasma, they are also exposed to random destruction as any other hematopoietic cells (21,22). Younger stress reticulocytes are removed from circulation much faster than normal reticulocytes, which may be connected with mechanical stability and membrane rigidity of reticulocytes (23), although there have been reports suggesting that in phlebotomy-induced anemia, the immature reticulocytes undergo normal *in-vivo* maturation without a change in lifespan (24,25). One might expect then violation of the precursor–successor relationship between reticulocytes and RBC due to the endogenous random destruction. This process could be modeled by introducing an endogenous conversion factor (CF) that would account for the fraction of reticulocytes that survived and become MRBC. In our model the destruction of reticulocytes was not included since the parameter CF would not be identifiable from the available data together with the remaining model parameters. Our simulations indicated (data not shown) that T_{RET} and CF were highly correlated, since increasing T_{RET} had the same effect on RBC predictions as decreasing CF, and consequently neither of these parameters would be reliably estimated simultaneously. Blood loss due to sampling for pharmacokinetic and pharmacodynamic measurements was not incorporated in our model. The total quantity of blood withdrawn during each day of study was less than 0.2% except for first 3 days where it was less than 1% and was not considered a significant perturbation of the erythropoiesis.

In summary, applying a simplistic assumption that all reticulocytes have the same lifespan and all mature to RBC, we were able to establish an explicit relationship between reticulocyte and RBC counts in plasma at any moment of time less than RBC life span. This precursor–successor relationship was used to predict RBC increase from reticulocyte counts due to rHu-EPO treatment. The predictions were significantly different from observations for rHu-EPO doses higher than 20 kIU leading to the hypothesis that rHu-EPO transiently increases the reticulocyte lifespan. We positively tested this hypothesis by proposing an empirical PD model that allowed us to estimate the increase in the mean reticulocyte lifespan distribution. Additionally, we were able to quantify the basic changes in the reticulocyte production rate following the rHu-EPO treatment. The results of the present study can be further utilized in building more mechanistic PKPD models of rHu-EPO stimulatory effect on RBC production.

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APPENDIX A

Derivation of the Precursor–successor Relationship Eq. 12

If the fixed lifespan T_{RET0} is assumed for all reticulocytes, then the RBC production is the delayed T_{RET0} reticulocyte production rate (11):

$$k_{out}(k) = k_R(t - T_{RET0}) \tag{25}$$

Thus, similarly to Eq. 11, one can write

$$\mathrm{MRBC}(t) = \int_{t-T_{\mathrm{RBC}}}^{t} k_{\mathrm{R}}(z - T_{\mathrm{RET0}})\mathrm{d}z \tag{26}$$

Changing the variables in the above integral $s = z - T_{\mathrm{RET0}}$ leads to

$$\mathrm{MRBC}(t) = \int_{t-T_{\mathrm{RET0}}-T_{\mathrm{RBC}}}^{t-T_{\mathrm{RET0}}} k_{\mathrm{R}}(s)\mathrm{d}s \tag{27}$$

The interval of integration in Eq. 27, $t - T_{\mathrm{RET0}} > s > t - T_{\mathrm{RET0}} - T_{\mathrm{RBC}}$, can be partitioned to the following subintervals: $t - T_{\mathrm{RET}} > s > t - 2T_{\mathrm{RET}}$, $t - 2T_{\mathrm{RET}} > s > t - 3T_{\mathrm{RET}}, \ldots,$ $t - NT_{\mathrm{RET}} > s > t - (N+1)T_{\mathrm{RET0}}$, where $N = \mathrm{INT}(t/T_{\mathrm{RET0}})$ is the integer describing how many times t is bigger than T_{RET0}. One can now decompose the integral in Eq. 27 into a sum of integrals over these subintervals and a remainder:

$$\mathrm{MRBC}(t) = \sum_{i=1}^{N} \int_{t-(i+1)\cdot T_{\mathrm{RET0}}}^{t-i\cdot T_{\mathrm{RET0}}} k_{\mathrm{R}}(s)\mathrm{d}s + \int_{t-T_{\mathrm{RBC}}-T_{\mathrm{RET0}}}^{t-(N+1)\cdot T_{\mathrm{RET0}}} k_{\mathrm{R}}(s)\mathrm{d}s \tag{28}$$

Equation 11 implies that for any integer i

$$\mathrm{RET}(t - i\cdot T_{\mathrm{RET}}) = \int_{t-(i+1)\cdot T_{\mathrm{RET0}}}^{t-i\cdot T_{\mathrm{RET0}}} k_{\mathrm{R}}(s)\mathrm{d}s \tag{29}$$

Since for $t/T_{\mathrm{RET}} < N+1$, the upper limit in the reminder integral in Eq. 28 is less than 0, and for $t \le T_{\mathrm{RBC}}$ the lower limit in this integral is less than 0 as well. We assume that prior to erythropoietin treatment the reticulocyte production was at the baseline level, $k_{\mathrm{R}}(s) = k_{\mathrm{R0}}$ for $s < 0$. Then the reminder integral in Eq. 28 is equal to

$$\int_{t-T_{\mathrm{RBC}}-T_{\mathrm{RET0}}}^{t-(N+1)\cdot T_{\mathrm{RET0}}} k_{\mathrm{R}}(z)\mathrm{d}z = T_{\mathrm{RBC}}\cdot k_{\mathrm{R0}} - N\cdot T_{\mathrm{RET0}}\cdot k_{\mathrm{R0}} \tag{30}$$

Taking into account the baseline Eqs. 4 and 5 yields

$$T_{\mathrm{RBC}}\cdot k_{\mathrm{R0}} - N\cdot T_{\mathrm{RET0}}\cdot k_{\mathrm{R0}} = \mathrm{MRBC}_0 - N\cdot \mathrm{RET}_0 \tag{31}$$

One can now combine Eqs. 28, 29, 30, 31, and obtain

$$\mathrm{MRBC}(t) - \mathrm{MRBC}_0 = \sum_{i=1}^{N} \mathrm{RET}(t - iT_{\mathrm{RET0}}) - \mathrm{RET}_0 \tag{32}$$

Because RBC is the sum of MRBC and RET, then

$$\Delta\mathrm{RBC}(t) = \Delta\mathrm{RET}(t) + \sum_{i=1}^{N} \Delta\mathrm{RET}(t - iT_{RET0}) \tag{33}$$

and Eq. 12 follows.

APPENDIX B

Derivation of equation for RET(t)

Taking into account Eq. 16 for the reticulocyte conversion rate $k_{\mathrm{out}}(t)$ one can integrate both sides of Eq. 1 from 0 to t and obtain

$$\mathrm{RET}(t) = R(0) + \int_0^t k_{\mathrm{R}}(z)\mathrm{d}z - \int_0^t k_{\mathrm{R}}(z - T_{\mathrm{RET0}})(1 - \chi(z - T_{\mathrm{RET0}}))\mathrm{d}z - \int_0^t k_{\mathrm{R}}(z - T_{\mathrm{RET}})\chi(z - T_{\mathrm{RET}})\mathrm{d}z \tag{34}$$

Changing the variables in the second integral $s = z - T_{\mathrm{RET0}}$ and in the third integral $s = z - T_{\mathrm{RET}}$ yields

$$\mathrm{RET}(t) = R(0) + \int_0^t k_{\mathrm{R}}(z)\mathrm{d}z - \int_{-T_{\mathrm{RET0}}}^{t-T_{\mathrm{RET0}}} k_{\mathrm{R}}(s)\mathrm{d}z + \int_{-T_{\mathrm{RET0}}}^{t-T_{\mathrm{RET0}}} k_{\mathrm{R}}(s)\chi(s)\mathrm{d}z - \int_{-T_{\mathrm{RET}}}^{t-T_{\mathrm{RET}}} k_{\mathrm{R}}(s)\chi(s)\mathrm{d}z \tag{35}$$

Since for $t < 0$ $k_{\mathrm{R}}(t) = k_{\mathrm{R0}}$ and $\chi(t) = 0$, the integrals in Eq. 35 can be further simplified to

$$\mathrm{RET}(t) = R(0) - T_{\mathrm{RET0}}k_{\mathrm{R0}} + \int_0^t k_{\mathrm{R}}(z)\mathrm{d}z - \int_0^{t-T_{\mathrm{RET0}}} k_{\mathrm{R}}(s)\mathrm{d}z + \int_0^{t-T_{\mathrm{RET0}}} k_{\mathrm{R}}(s)\chi(s)\mathrm{d}z - \int_0^{t-T_{\mathrm{RET}}} k_{\mathrm{R}}(s)\chi(s)\mathrm{d}z \tag{36}$$

The reticulocytes at time $t = 0$ are at steady-state, therefore Eq. 4 implies that the first and second term in Eq. 36 cancel each other out. If the first integral is combined with the second, and the third with the fourth, then one obtains

$$\mathrm{RET}(t) = \int_{t-T_{\mathrm{RET0}}}^{t} k_{\mathrm{R}}(z)\mathrm{d}z + \int_{t-T_{\mathrm{RET}}}^{t-T_{\mathrm{RET0}}} k_{\mathrm{R}}(z)\chi(z)\mathrm{d}z \tag{37}$$

which is the exact form of Eq. 17. Assuming the following relationships between the time parameters

$$T_{\mathrm{RET0}} < T_{\mathrm{TRET}}, T_0 + T_{\mathrm{RET}} < T_1, \text{and } T_1 + T_{\mathrm{RET}} < T_2 \tag{38}$$

they can be ordered as follows $T_0 < T_0 + T_{\mathrm{RET0}} < T_0 + T_{\mathrm{RET}} < T_1 < T_1 + T_{\mathrm{RET0}} < T_1 + T_{\mathrm{RET}} < T_2 < T_2 + T_{\mathrm{RET0}}$.

Consequently, an arbitrary t value must fall into one of the following intervals:

$t \le T_0$, then

$$\mathrm{RET}(t) = \mathrm{RET}_0 \tag{39}$$

$T_0 < t \le T_0 + T_{\mathrm{RET0}}$, then

$$\begin{aligned}\mathrm{RET}(t) &= \int_{t-T_{\mathrm{RET0}}}^{t} k_{\mathrm{R}}(z)\mathrm{d}z \\ &= \int_{t-T_{\mathrm{RET0}}}^{T_0} k_{\mathrm{R}}(z)\mathrm{d}z + \int_{T_0}^{t} k_{\mathrm{R}}(z)\mathrm{d}z \\ &= k_{\mathrm{R0}}(T_0 - t + T_{\mathrm{RET0}}) + k_{\mathrm{R1}}(t - T_0)\end{aligned} \tag{40}$$

$T_0 + T_{\mathrm{RET0}} < t \le T_0 + T_{\mathrm{RET}}$, then

$$\begin{aligned}\mathrm{RET}(t) &= \int_{t-T_{\mathrm{RET0}}}^{T_0} k_{\mathrm{R}}(z)\mathrm{d}z + \int_{T_0}^{t} k_{\mathrm{R}}(z)\mathrm{d}z \\ &\quad + \int_{T_0}^{t-T_{\mathrm{RET0}}} k_{\mathrm{R}}(z)\chi(z)\mathrm{d}z \\ &= \int_{T_0}^{t} k_{\mathrm{R}}(z)\mathrm{d}z = k_{\mathrm{R1}}(t - T_0)\end{aligned} \tag{41}$$

$T_0 + T_{\mathrm{RET}} < t \le T_1$, then

$$\mathrm{RET}(t) = k_{\mathrm{R1}} T_{\mathrm{RET0}} + k_{\mathrm{R1}}(T_{\mathrm{RET}} - T_{\mathrm{RET0}}) = k_{\mathrm{R1}} T_{\mathrm{RET}} \tag{42}$$

$T_1 < t \le T_1 + T_{\mathrm{RET0}}$, then

$$\begin{aligned}\mathrm{RET}(t) &= \int_{t-T_{\mathrm{RET0}}}^{T_1} k_{\mathrm{R}}(z)\mathrm{d}z + \int_{T_1}^{t} k_{\mathrm{R}}(z)\mathrm{d}z \\ &\quad + \int_{t-T_{\mathrm{RET}}}^{t-T_{\mathrm{RET0}}} k_{\mathrm{R}}(z)\chi(z)\mathrm{d}z \\ &= \int_{t-T_{\mathrm{RET}}}^{T_1} k_{\mathrm{R}}(z)\mathrm{d}z + \int_{T_1}^{t} k_{\mathrm{R}}(z)\mathrm{d}z \\ &= k_{\mathrm{R1}}(T_1 - t + T_{\mathrm{RET}}) + k_{\mathrm{R2}}(t - T_1)\end{aligned} \tag{43}$$

$T_1 + T_{\mathrm{RET0}} < t \le T_1 + T_{\mathrm{RET}}$, then

$$\begin{aligned}\mathrm{RET}(t) &= \int_{t-T_{\mathrm{RET0}}}^{t} k_{\mathrm{R}}(z)\mathrm{d}z + \int_{t-T_{\mathrm{RET}}}^{T_1} k_{\mathrm{R}}(z)\mathrm{d}z \\ &= k_{\mathrm{R2}} T_{\mathrm{RET0}} + k_{\mathrm{R1}}(T_1 - t + T_{\mathrm{RET}})\end{aligned} \tag{44}$$

$T_1 + T_{\mathrm{RET}} < t \le T_2$, then

$$\mathrm{RET}(t) = k_{\mathrm{R2}} T_{\mathrm{RET0}} \tag{45}$$

$T_2 < t \le T_2 + T_{\mathrm{RET0}}$, then

$$\begin{aligned}\mathrm{RET}(t) &= \int_{t-T_{\mathrm{RET0}}}^{T_2} k_{\mathrm{R}}(z)\mathrm{d}z + \int_{T_2}^{t} k_{\mathrm{R}}(z)\mathrm{d}z \\ &= k_{\mathrm{R2}}(T_2 - t + T_{\mathrm{RET0}}) + k_{\mathrm{R0}}(t - T_2)\end{aligned} \tag{46}$$

$T_2 + T_{\mathrm{RET0}} \le t$, then

$$\mathrm{RET}(t) = k_{\mathrm{R0}} T_{\mathrm{RET0}} \tag{47}$$

Arranging terms in Eqs. 39, 40, 41, 42, 43, 44, 45, 46, 47 yields Eq. 10. A similar derivation holds for Eq. 20, except that one needs to consider the cases determined by the intervals $T_0 < T_1 < T_2 < T_{\mathrm{RBC}}$ and use Eq. 8.

APPENDIX C

NONMEM control stream and data file for estimation of T_{RET0} from Eq. 12

```
$PROB Estimation of TRET
$INPUT ID TIME CMT MDV DV RET RBC DOSE
$DATA C:\data_1041.csv IGNORE=#
$PRED
TRET = THETA(1)*EXP(ETA(1))

"OPEN(2,FILE='C:\nmv\run\fdata')
"REWIND 2
"DO WHILE (.NOT.EOF(2))
" READ(2,*) X1,X2,X3,X4,X5,X6,X7,X8
" IF(X1.EQ.ID.AND.CMT.EQ.1) THEN
" IF (X2.EQ.0) RT0=X6
" IF (X2.EQ.2) RT2=X6
" IF (X2.EQ.3) RT3=X6
" IF (X2.EQ.4) RT4=X6
" IF (X2.EQ.5) RT5=X6
" IF (X2.EQ.6) RT6=X6
" IF (X2.EQ.7) RT7=X6
" IF (X2.EQ.8) RT8=X6
" IF (X2.EQ.9) RT9=X6
" IF (X2.EQ.11) RT11=X6
" IF (X2.EQ.13) RT13=X6
" IF (X2.EQ.15) RT15=X6
" IF (X2.EQ.17) RT17=X6
" IF (X2.EQ.19) RT19=X6
" IF (X2.EQ.21) RT21=X6
" IF (X2.EQ.23) RT23=X6
" IF (X2.EQ.25) RT25=X6
" IF (X2.EQ.27) RT27=X6
" IF (X2.EQ.28) RT28=X6
" IF (X2.EQ.31) RT31=X6
" IF (X2.EQ.32) RT32=X6
" IF (X2.EQ.34) RT34=X6
" IF (X2.EQ.35) RT35=X6
" IF (X2.EQ.40) RT40=X6
" IF (X2.EQ.42) RT42=X6
" IF (X2.EQ.0) RBC0=X7
"ENDIF
"ENDDO
"CLOSE(2)
" RB=RBC0
```

```
" IF(DOSE.GT.0) THEN
" I=0
" DO WHILE (TIME-I*TRET.GE.0.0)
" X=TIME-I*TRET
" IF (0.LT.X.AND.X.LE.2) RB=RB+RT0+(RT2-RT0)/
(2-0)*(X-0)-RT0
" IF (2.LT.X.AND.X.LE.3) RB=RB+RT2+(RT3-RT2)/
(3-2)*(X-2)-RT0
" IF (3.LT.X.AND.X.LE.4) RB=RB+RT3+(RT4-RT3)/
(4-3)*(X-3)-RT0
" IF (4.LT.X.AND.X.LE.5) RB=RB+RT4+(RT5-RT4)/
(5-4)*(X-4)-RT0
" IF (5.LT.X.AND.X.LE.6) RB=RB+RT5+(RT6-RT5)/
(6-5)*(X-5)-RT0
" IF (6.LT.X.AND.X.LE.7) RB=RB+RT6+(RT7-RT6)/
(7-6)*(X-6)-RT0
" IF (7.LT.X.AND.X.LE.8) RB=RB+RT7+(RT8-RT7)/
(8-7)*(X-7)-RT0
" IF (8.LT.X.AND.X.LE.9) RB=RB+RT8+(RT9-RT8)/
(9-8)*(X-8)-RT0
" IF (9.LT.X.AND.X.LE.11) RB=RB+RT9+(RT11-RT9)/
(11-9)*(X-9)-RT0
" IF (11.LT.X.AND.X.LE.13) RB=RB+RT11+(RT13-RT11)/
(13-11)*(X-11)-RT0
" IF (13.LT.X.AND.X.LE.15) RB=RB+RT13+(RT15-RT13)/
(15-13)*(X-13)-RT0
" IF (15.LT.X.AND.X.LE.17) RB=RB+RT15+(RT17-RT15)/
(17-15)*(X-15)-RT0
" IF (17.LT.X.AND.X.LE.19) RB=RB+RT17+(RT19-RT17)/
(19-17)*(X-17)-RT0
" IF (19.LT.X.AND.X.LE.21) RB=RB+RT19+(RT21-RT19)/
(21-19)*(X-19)-RT0
" IF (21.LT.X.AND.X.LE.23) RB=RB+RT21+(RT23-RT21)/
(23-21)*(X-21)-RT0
" IF (23.LT.X.AND.X.LE.25) RB=RB+RT23+(RT25-RT23)/
(25-23)*(X-23)-RT0
" IF (25.LT.X.AND.X.LE.27) RB=RB+RT25+(RT27-RT25)/
(27-25)*(X-25)-RT0
" IF (27.LT.X.AND.X.LE.28) RB=RB+RT27+(RT28-RT27)/
(28-27)*(X-27)-RT0
" IF (28.LT.X.AND.X.LE.31) RB=RB+RT28+(RT31-RT28)/
(31-28)*(X-28)-RT0
" IF (31.LT.X.AND.X.LE.32) RB=RB+RT31+(RT32-RT31)/
(32-31)*(X-31)-RT0
" IF (32.LT.X.AND.X.LE.34) RB=RB+RT32+(RT34-RT32)/
(34-32)*(X-32)-RT0
" IF (34.LT.X.AND.X.LE.35) RB=RB+RT34+(RT35-RT34)/
(35-34)*(X-34)-RT0
" IF (35.LT.X.AND.X.LE.40) RB=RB+RT35+(RT40-RT35)/
(40-35)*(X-35)-RT0
" IF (40.LT.X.AND.X.LE.42) RB=RB+RT40+(RT42-RT40)/
(42-40)*(X-40)-RT0
" I=I+1
" ENDDO
" ENDIF

IPRED=RB+ERR(1)
Y = IPRED
IRES = DV - IPRED
$THETA (0,,15) ;TRET
$OMEGA 0.00 FIX
$SIGMA 0.02

$EST NSIGDIG=3 MAX=9999 PRINT=1 NOABORT
POSTHOC METHOD=1
$COV PRINT=E
$TABLE ID TIME CMT IPRED IRES DOSE RET
NOPRINT FILE=C:\1041.txt
```

The following file data_1041.txt contains records for Subject 1041. The missing values for RET were calculated by the linear interpolation of the neighboring reticulocyte measurements

#ID	TIME	CMT	MDV	DV	RET	RBC	DOSE
1041	0	1	0	5.145	0.054	5.145	40
1041	2	1	0	5.55	0.072	5.55	40
1041	3	1	0	5.49	0.135	5.49	40
1041	4	1	0	5.36	0.192	5.36	40
1041	5	1	0	5.2	0.253	5.2	40
1041	6	1	0	5.26	0.245	5.26	40
1041	7	1	0	5.5	0.291	5.5	40
1041	8	1	0	5.4	0.349	5.4	40
1041	9	1	0	5.69	0.218	5.69	40
1041	11	1	0	5.44	0.132	5.44	40
1041	13	1	0	5.37	0.103	5.37	40
1041	15	1	0	5.27	0.092	5.27	40
1041	17	1	0	5.45	0.070	5.45	40
1041	19	1	0	5.55	0.093	5.55	40
1041	21	1	0	5.39	0.072	5.39	40
1041	23	1	0	5.49	0.083	5.49	40
1041	25	1	0	5.55	0.084	5.55	40
1041	27	1	1	0	0.108	0	40
1041	28	1	0	5.49	0.120	5.49	40
1041	31	1	1	0	0.111	0	40
1041	32	1	1	0	0.108	0	40
1041	34	1	1	0	0.101	0	40
1041	35	1	1	0	0.098	0	40
1041	40	1	1	0	0.082	0	40
1041	42	1	0	5.22	0.076	5.22	40

APPENDIX D

NONMEM control stream for estimation of T_0, ΔT, ΔT_1, Δk_{R1}, and Δk_{R2}

```
$PROB RHUEPO EFFECT ON RET AND RBC
$INPUT ID TIME CMT MDV DV RET RBC DOSE
$DATA C:\data_1034.csv IGNORE = #
$PRED

T0 = THETA(1)*EXP(ETA(1))
DT1 = THETA(2)
DT = THETA(3)
DT2 = THETA(4)
DKR1 = THETA(5)
DKR2 = THETA(6)

"OPEN(2,FILE='C:\nmv\run\fdata')
"REWIND 2
"DO WHILE (.NOT.EOF(2))
" READ(2,*) X1,X2,X3,X4,X5,X6,X7,X8
" IF(X1.EQ.ID.AND.CMT.EQ.1) THEN
" IF (X2.EQ.0) THEN
" RT0=X6
```

```
" RBC0=X7
" TT=RT0/(RBC0−RT0)*120.0
" ENDIF
" ENDIF
"ENDDO
" CLOSE(2)
KR0=RT0/TT
KR1=KR0+DKR1
KR2=KR0−DKR2
T1=T0+DT1
T2=T1+DT2
TR=TT+DT
CH0=0
CH1=1
CH2=0
X=TIME
IT1=0
IT2=0
IT3=0
IT4=0
IF (X.LE.T0) IT1=KR0*X
IF (X.GT.T0.AND.X.LE.T1) IT1=KR1*(X−T0)+KR0*T0
IF (X.GT.T1.AND.X.LE.T2) IT1=KR2*(X−T1)+KR1*
(T1−T0)+KR0*T0
IF (X.GT.T2) IT1=KR0*(X−T2)+KR2*(T2−T1)+ KR1*
(T1−T0)+KR0*T0
X=TIME−TT
IF (X.LE.T0) IT2=KR0*X
IF (X.GT.T0.AND.X.LE.T1) IT2=KR1*(X−T0)+ KR0*T0
IF (X.GT.T1.AND.X.LE.T2) IT2=KR2*(X−T1)+KR1*
(T1−T0)+KR0*T0
IF (X.GT.T2) IT2=KR0*(X−T2)+KR2*(T2−T1)+KR1*
(T1−T0)+KR0*T0
X=TIME−TT
IF (X.LE.T0) IT3=KR0*CH0*X
IF (X.GT.T0.AND.X.LE.T1) IT3=KR1*CH1*(X−T0)+
KR0*CH0*T0
IF (X.GT.T1.AND.X.LE.T2) THEN
IT3=KR2*CH2*(X−T1)+KR1*CH1*(T1−T0)+
KR0*CH0*T0
ENDIF
IF (X.GT.T2) THEN
IT3=KR0*CH0*(X−T2)+KR2*CH2*(T2−T1)+KR1*CH1*
(T1−T0)+KR0*CH0*T0
ENDIF
X=TIME−TR
IF (X.LE.T0) IT4=KR0*CH0*X
IF (X.GT.T0.AND.X.LE.T1) IT4=KR1*CH1*(X−T0)+
KR0*CH0*T0
IF (X.GT.T1.AND.X.LE.T2) THEN
IT4=KR2*CH2*(X−T1)+KR1*CH1*(T1−T0)+
KR0*CH0*T0
ENDIF
IF (X.GT.T2) THEN
IT4=KR0*CH0*(X−T2)+KR2*CH2*(T2−T1)+KR1*CH1*
(T1−T0)+KR0*CH0*T0
ENDIF
R=IT1−IT2+IT3−IT4
RB=IT1−KR0*TIME+RBC0

IPRED=0
IF(CMT.EQ.1) IPRED=RB+ERR(1)
IF(CMT.EQ.2) IPRED=R+ERR(2)
Y = IPRED
IRES = DV − IPRED

$THETA
(0,1.5,5) ;T0
(0,7.0 ,20) ;DT1
(0,2,20) ;DT
42 FIX ;DT2
(0,0.04, 0.1) ;DKR1
(0,0.01,0.05) ;DKR2

$OMEGA 0.0 FIX
$SIGMA
0.01 ; RBC
0.01 ; RET
$ESTIMATION NSIGDIG=3 MAX=999 PRINT=1
NOABORT POSTHOC METHOD=1
$COV PRINT=E

$TABLE ID TIME CMT IPRED DOSE RET RBC
NOPRINT
FILE=C:\1034.txt
```

#ID	TIME	CMT	MDV	DV	RET	RBC	DOSE
1034	0	1	0	4.925	0.079	4.925	160
1034	0	2	0	0.079	0.079	4.925	160
1034	2	1	0	5.38	0.126	5.38	160
1034	2	2	0	0.126	0.126	5.38	160
1034	3	1	0	5.59	0.149	5.59	160
1034	3	2	0	0.149	0.149	5.59	160
1034	4	1	0	5.49	0.189	5.49	160
1034	4	2	0	0.189	0.189	5.49	160

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