Pharmacokinetic and Pharmacodynamic Modeling of Recombinant Human Erythropoietin after Intravenous and Subcutaneous Administration in Rats

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Received July 21, 2006; accepted September 12, 2006

ABSTRACT

The pharmacokinetics (PK) and pharmacodynamics (PD) of recombinant human erythropoietin (rHuEPO) were studied in rats after single i.v. and s.c. administration at three dose levels (450, 1350, and 4050 IU/kg). The plasma concentrations of rHuEPO and its erythropoietic effects including reticulocyte (RET), red blood cell (RBC), and hemoglobin (Hb) levels were determined. A two-compartment model with dual input rate and nonlinear disposition was used to characterize the PK of rHuEPO. The catenary indirect response model with several compartments reflecting the bone marrow and circulating erythropoietic cells was applied. The s.c. doses exhibited slow absorption ($T_{\text{max}} = 12 \text{ h}$) and incomplete bioavailability ($F = 0.59$). In placebo groups, RBC and Hb values gradually increased over time with growth of the rats, and the changes in the baselines monitored from 8 to 32 weeks of age were described by a nonlinear growth function. All doses resulted in dose-dependent increases in RET counts followed by an immediate decline below the baseline at around 6 days and returned to the predose level in 21–24 days after dosing. A subsequent steady increase of RBC and Hb levels followed and reached peaks at 6 days. A tolerance phenomenon observed at all dose levels was modeled by a negative feedback inhibition with the relative change in Hb level. The PK/PD model well described the erythropoietic effects of rHuEPO as well as tolerance, thereby yielding important PD parameters ($S_{\text{max}} = 1.87$ and $SC_{\text{ld}} = 65.37 \text{ mIU/ml}$) and mean lifespans of major erythropoietic cell populations in rats.

Erythropoietin (EPO) is a glycoprotein hormone (30.5 kDa) produced in adult kidneys, and it is the major regulator of red cell production. Tissue hypoxia is the primary stimulus of the production of endogenous EPO. EPO binds to its receptors on the surface of erythroid progenitors in bone marrow, leading to their survival, proliferation, and differentiation, which in turn produces an increase in RBC and hemoglobin (Hb) concentrations (Fisher, 2003). Conversely, an excessive increase in RBC mass suppresses erythropoiesis to prevent blood from becoming more viscous, leading to the possibility of thrombosis and stroke. One study showed that transfusion polycythemia depressed bone marrow activity and reduced production of RBC in normal subjects (Birkhill et al., 1951). The negative feedback control in erythropoiesis has also been observed in humans after the subsequent rises of RBC and Hb following rHuEPO administration (Ramakrishnan et al., 2004). The exact mechanism and the primary regulators responsible for the counter-regulation are, however, not clearly elucidated.

Comprehensive PK/PD models have been developed to quantitatively account for the pharmacokinetics and erythropoietic effects of rHuEPO (Ramakrishnan et al., 2003, 2004). The models depict nonlinear disposition kinetics and absorption kinetics mainly characterized by prolonged absorption and variable, incomplete bioavailability upon s.c. administration. Because erythropoiesis involves a series of differentiation stages from pluripotent hematopoietic stem cells through the mature erythrocyte, an array of hematological changes can be observed after rHuEPO administration. The primary response variables considered include reticulocyte, RBC, and Hb levels, and these variables are quantitatively described by a mathematical model considering lifespans of cell populations and the basis of physiological mechanisms. The PK/PD analysis of rHuEPO increases our current understanding about this complex system and also leads to questions that still need to be answered.

With the present study we seek further understanding of

**ABBREVIATIONS:** EPO, erythropoietin; RBC, red blood cell; Hb, hemoglobin; rHuEPO, recombinant human erythropoietin; PK, pharmacokinetics; PD, pharmacodynamics; RET, reticulocyte; MCH, mean corpuscular hemoglobin; WBC, white blood cell.
the regulation of erythropoiesis and the erythropoietic effects of rHuEPO using extensive experimental data obtained from rats. Often overlooked is the fact that excess blood removal resulting from intensive sampling can naturally stimulate erythropoiesis, which has a potential to interfere in the assessment of the effects of exogenously administered rHuEPO. This is of considerable importance in the avoidance of biased or imprecise results when animals of small size such as rats are used and could be one reason why few pharmacodynamic studies of rHuEPO have been performed in rats. In fact, even the data available in the literature are not sufficient to be analyzed by a PK/PD modeling approach because of limited sample collections. The purpose of this study is to characterize the relationship of an array of erythropoietic responses upon rHuEPO administration using a mechanism-based PK/PD model and to address general concerns in experiments in rats to assess the PK and PD of rHuEPO.

Methods and Materials

Animals. All studies were approved by the Institutional Animal Care and Use Committee of the University at Buffalo. Male Wistar rats with weights ranging from 275 to 300 g were purchased from Charles River Laboratories, Inc. (Raleigh, NC). All animals had free access to food and water and were maintained on a 12:12-h light/dark cycle. All animals were acclimatized for 1 week before the initiation of the studies.

Experimental Design. Recombinant human erythropoietin (EPOGEN, 10,000 units/ml; Amgen Inc., Thousand Oaks, CA) was diluted immediately before injection using saline for injection (B. Braun Medical Inc., Irvine, CA) containing 0.25% bovine serum albumin (Sigma-Aldrich, St. Louis, MO). Seventy-two rats were divided into eight groups (three i.v. doses and one control; three s.c. doses and one control) on the basis of equivalent mean values of RBC, Hb, RET, and body weight. Each group (doses and one control) was prepared so that the individual mean values of the cytokine ELISA (R&D Systems Inc., Minneapolis, MN). Endogenous EPO in rats was not detected. Standard curves ranged from 0 to 200 mIU/ml. The lower limit of detection was <1 mIU/ml, and the lower limit of quantification was 2 mIU/ml. The coefficient of variation of the assay over the range of measured concentrations was <10%. Plasma samples with rHuEPO concentrations >200 mIU/ml were diluted with the diluents provided by the manufacturer. General procedures followed the manufacturer's instructions. Plates were read at OD450 to OD650 on a SpectraMax 190 ELISA plate reader (Molecular Devices, Sunnyvale, CA). All samples for rHuEPO concentrations were analyzed on the same day to reduce variability.

Hematological Measurements. A Cell-Dyn 1700 (Abbott Laboratories, Abbott Park, IL) was used for following hematological tests: RBC count (10⁶ per microliter), Hb concentration (grams per deciliter), mean corpuscular hemoglobin (MCH) (picograms per cell), platelet count (10⁵ per microliter), white blood cell count (WBC) (10³ per microliter), and WBC count differential. Hematological parameters in the blood with EDTA were analyzed within 4 h of blood collection.

RET counts were enumerated using flow cytometry (FACS Calibur; BD Biosciences, Franklin Lakes, NJ) with thiazole orange stain. All procedures were based on the manufacturer's instructions. In brief, 5 μl of EDTA-treated whole blood was added to tubes containing 1 ml of staining solution with thiazole orange (Becton-Dickinson) and incubated for 60 min in the dark at room temperature. In addition, an additional 5 μl of the whole blood was added into a tube containing 1 ml of phosphate-buffered saline with 0.1% sodium azide followed by incubation. This sample served as an unstained control for autofluorescence background. Flow cytometry analysis was then performed with the detection of forward scatter, side scatter, and green fluorescence. Data analysis involved gating the RBC population on a scattergram (forward scatter versus side scatter) to exclude debris, platelets, and WBCs, acquiring 50,000 events in the RBC gate. Green fluorescence from thiazole orange bound to RNA residuals in reticulocyte was determined as a percentage of positive events (%RET) in the region of RBCs by setting a threshold using an unstained control. The absolute RET counts were then calculated by multiplying %RET by the RBC counts obtained from the automated hematology analyzer.

PK/PD Model. A PK/PD model for rHuEPO in monkeys (Ramakrishnan et al., 2003) and in humans (Ramakrishnan et al., 2004) was developed previously in our laboratory. The PK and PD data in rats were analyzed with modifications of the model as shown in Fig. 1.

Pharmacokinetic Model. The plasma concentration versus time profiles of rHuEPO were fitted to a two-compartment model with parallel elimination involving linear first-order elimination (k_e) and Michaelis-Menten saturable kinetics (V_{max} and K_m). Differential equations for the i.v. data can be written as

\[
\frac{dA_{Epo}}{dt} = -\frac{V_{max} \times A_{Epo}}{K_m \times V_p + A_{Epo}} \times (k_e + k_p) \times A_{Epo} + k_{ip} \times A_T
\]

where \(A_{Epo}(0) = \text{Dose}\) (1)

\[
\frac{dA_T}{dt} = k_{ip} \times A_{Epo} - k_{ip} \times A_T \quad \text{where } A_T(0) = 0
\]

where \(A_{Epo}\) and \(A_T\) are the amounts of rHuEPO in the central and peripheral compartments, \(V_p\) is the volume of distribution for the central compartment, and \(k_{ip}\) and \(k_{ip}\) are intercompartmental rate constants.

The absorption kinetics after s.c. administration was assumed to follow dual absorption input as described previously (Ramakrishnan et al., 2003).
et al., 2003), where rHuEPO was absorbed into the blood via a zero-order process (\(k_d\)) from times \(t < \tau\) followed by a first-order process (\(k_d\)) from the injection site:

\[
\frac{dA_{Epo}}{dt} = \text{Input}(t) - \frac{V_{max} \times A_{Epo}}{K_m + V_p + A_{Epo}} - (k_d + k_{in}) \times A_{Epo} + k_{ip} \times A_T
\]

\[
\frac{dA_T}{dt} = k_{pl} \times A_{Epo} - k_{ip} \times A_T \quad \text{where} \ A_{Epo}(0) - A_T(0) = 0.
\]

\[
\text{Input}(t) = \Theta(t - \tau) \times \frac{F \times (1 - Fr) \times \text{Dose}}{\tau} + \Theta(t - \tau) \times k_d \times F \times \text{Dose} \times e^{-k_d(t - \tau)}
\]

where \(F\) denotes bioavailability of s.c. administered rHuEPO, \(Fr\) indicates the fraction absorbed via the first-order process (\(k_d\)), and \(\Theta(z)\) is the jump function equal to 1 for \(z > 0\) and 0 otherwise.

**Pharmacodynamic Model.** Erythropoiesis is the process whereby a fraction of hematopoietic stem cells becomes committed to the red cell lineage, first forming burst-forming unit-erythrocytes and then colony-forming unit-erythrocytes, erythroblasts, reticulocytes, and ultimately the mature RBCs. EPO is primarily responsible for the proliferation and differentiation of erythroid progenitors into erythrocytes. Erythroid cells respond to EPO in a stage-specific manner based on the presence of the erythropoietin receptor. Differentiation from progenitor cells (i.e., colony-forming unit-erythrocytes) to erythroblasts is highly dependent on EPO, whereas differentiation afterward is no longer EPO-dependent (Fisher, 2003).

A pharmacodynamic model for the erythropoietic effects of rHuEPO can be described on the basis of the combination of cell production and loss model (i.e., cell lifespan concept) and indirect response model (Krzyzanski et al., 1999). The proposed model shown in Fig. 1 depicts the process of erythropoiesis. The first precursor compartment (P1) represents all the earliest progenitor cells, which, in turn, are converted to erythroblasts (P2), RETs, and mature RBCs (RBC_M). The average times taken for progenitor cells to be converted into erythroblasts, for erythroblasts into RETs, and for RETs into RBCs were accounted for by times \(T_{P1}\), \(T_{P2}\), and \(T_{RBC}\). The \(T_{RBC}\) indicates the mean lifespan of RBCs. A rebound phenomenon was observed in RETs, falling below the baseline after the peak effect, and was captured with the feedback regulation driven by the hemoglobin change relative to its baseline (\(\Delta Hb\)). The differential equations for the RET and RBC_M counts are as follows:

\[
\frac{dRET}{dt} = k_{in} \times S(t - T_{P1} - T_{P2}) \times S(t - T_{P2}) \times I(t - T_{P1} - T_{P2}) - k_{in} \times S(t - T_{P1} - T_{P2} - T_{RET}) \times S(t - T_{P2} - T_{RET}) \times I(t - T_{P1} - T_{P2} - T_{RET})
\]

\[
\frac{dRBC_M}{dt} = k_{in} \times S(t - T_{P1} - T_{P2} - T_{RET}) \times S(t - T_{P2} - T_{RET}) \times I(t - T_{P1} - T_{P2} - T_{RET} - T_{RBC})
\]

\[
S(t) = \left(1 - \frac{S_{\text{max}} \times A_{Epo}(t)/V_p}{SC_{50} + A_{Epo}(t)/V_p}\right) \quad \text{and} \quad I(t) = \left(1 - \frac{I_{\text{max}} \times \Delta Hb(t)}{IC_{50} + \Delta Hb(t)}\right)
\]

where \(S_{\text{max}}\) is the maximal possible stimulation of responses by rHuEPO, and \(SC_{50}\) is the plasma concentration of rHuEPO producing half of the maximal stimulation. The endogenous EPO was not included in the model because the baseline level of EPO in normal rats was below the limit of quantification of the assay. Therefore, it was assumed that the endogenous EPO is negligible. Because the RBC count measured by a conventional hematology analyzer includes RETs as well as mature RBCs in the blood, the observed RBC data were fitted to a sum of RET and RBC_M to account for a portion of RETs in the measured RBC level:

\[
RBC = RBC_0 + \Delta \text{RET} + \Delta \text{RBC}_M
\]
where RBC₀ indicates the RBC baseline, and ΔRET and ΔRBCM represent changes from the baseline of RET (ΔRET = RET - RET₀) and RBCM (ΔRBCM = RBCM - RBCM₀). Inhibition by the Hb change (ΔHb = Hb - Hb₀) on the production rate of progenitor cells was modeled using an inhibitory function with I_{max} function of 1. The values of T₁, T₂, and T₃ were estimated by the model. The mean RBC lifespan (T_RBC) of Wistar rats was reported to be 59.8 ± 2.2 days (Derelanko, 1987) and fixed during the modeling process as 60 days. The kₘ was estimated as a secondary parameter based on the relationship kₘ = RET₅₀/T₃ RET.

MCH, which indicates hemoglobin content per cell, was obtained by the hematology analyzer. This value was used to calculate the hemoglobin concentration as a product of RBC and MCH based on (Wintrobe, 2003):

\[
\text{Hb}(\text{g/dl}) = \frac{\text{MCH}(\text{pg/cell}) \times \text{RBC}(\times 10^6 \text{cells/μl})}{10}
\]  
(8)

**Hematological Baselines.** The baseline values of erythrocyte indices such as RBCs, Hb, and MCH changed significantly over time, and these changes needed to be considered in the final modeling processes. For this purpose, data from the placebo groups and the negative control group were pooled together and modeled with empirical functions to address overall decreases or increases in these response variables. The Gompertz equation (Laird, 1964) was applied to account for the overall increase in RBC baseline (RBC₀):

\[
\text{RBC}_t = \text{RBC}_0 \times e^{k_{syn} - \sigma_{ret} \times t}
\]  
(9)

where kₕ is the growth constant, α is the retarding constant, and RBC₀ is the value of the measured RBC count at the starting point. MCH reflects the content of Hb per cell and was shown to decrease over time. This was modeled by the indirect response model:

\[
\frac{d \text{MCH}}{dt} = k_{syn}(t) - k_{int} \times \text{MCH} \quad \text{where MCH}(0) = \text{MCH}_0
\]  
(10)

where the change in MCH is controlled by a time-dependent change in synthesis process [k_{syn}(t)], a constant first-order elimination process [k_{int}], and MCH₀ is the observed value at the beginning of the studies. It was assumed that the rate of production k_{syn}(t) gradually decreases and reaches its minimal value (k_{syn, min}). This can be written as

\[
\frac{dk_{syn}}{dt} = k_d \times \left[1 - \frac{k_{syn}}{k_{syn, min}}\right] \times k_{syn} \quad \text{where k}_{syn}(0) = k_{syn0}
\]  
(11)

where the change in k_{syn}(t) over time is governed by a first-order process (k_d). The initial value of k_{syn} (k_{syn0}) was estimated. In addition, it was observed that the MCH increased after rHuEPO administration in a dose-dependent manner in the treatment groups. This was characterized using the nonlinear stimulatory function (S_{MCH} max/MCH and S_{50,MCH}) by rHuEPO concentration according to

\[
\frac{d \text{MCH}}{dt} = k_{syn}(t) \times \left(1 + \frac{S_{max,MCH} \times A_{Epo}(t/V_T)}{S_{50,MCH} + A_{Epo}(t/V_T)}\right) - k_{int} \times \text{MCH}
\]  
(12)

where MCH(t) = MCH₀

**Data Analysis.** All samples were pooled, and the mean data were used for all data analysis. Each drug concentration versus time profile was first analyzed using a noncompartmental technique by WinNonlin (Pharsight Corporation, Cary, NC) to determine the proportionality of drug exposure to dose. The PK data from three single i.v. doses and three single s.c. doses were first fitted to eqs. 1 to 4. The time courses of RET and RBC counts after rHuEPO administration at all dose levels were analyzed simultaneously with fixed PK profiles. The expected levels of Hb were calculated as eq. 8. Modeling for the baselines was done separately from the analysis for the treatment effects and the related parameters were fixed during PD analysis. All of the fittings were performed using the ADAPT II program (D’Argenio and Schumitzky, 1997) with the maximal likelihood method. The variance model used is as follows:

\[
V_i = \sigma_1^2 \times Y_i^{\alpha_2}
\]  
(13)

where \(V_i\) is the variance of the i-th data point, \(\sigma_1\) and \(\sigma_2\) are the variance model parameters, and \(Y_i\) represents the i-th model predicted value.

**Results**

**Experimental Approach.** The erythropoiesis process can be stimulated by blood loss from frequent and repeated sampling, and subsequent increases in erythrocyte indices can follow (Chapel et al., 2000; Criswell et al., 2000). Preliminary studies were conducted to address these concerns in the experimental design that allows measuring PK and PD determinants of rHuEPO in rats simultaneously without artifacts. In this study, each group (n = 9) consisted of three subgroups of three rats, and blood samples were drawn from three subgroups in a rotating manner. Combining all data from the three subgroups (e.g., 10 time points/subgroup) produced a complete set of time profiles of rHuEPO concentrations as well as all of its responses for each dose level. To avoid a bias from variation in erythrocyte indices between subgroups, two of the subgroups were always sampled together at a given time point. There was no notable hematological perturbation by bleeding in control groups, and the mean values of hematological variables between subgroups were comparable (data not shown). Therefore, we confirmed that this design allowed for simultaneous determination of PK and PD data of rHuEPO with minimal variability between subgroups.

**Pharmacokinetics.** The concentration versus time profiles for the various i.v. and s.c. doses are shown in Fig. 2. The noncompartmentally calculated parameters from these profiles are listed in Table 1. Nonlinear PK behavior of rHuEPO in rats was evidenced by a dose-dependent decrease in apparent clearance and the terminal slope of rHuEPO with corresponding dose-dependent increases in the area under the curve. The initial volume of distribution (57–59 ml/kg) determined as \(D_{IV} \times C_{IV0}\) by the initial drug concentration \(C_{IV0}\) and the IV dose \(D_{IV} \) was smaller compared with the steady-state volume of distribution (115–118 ml/kg), indicating the need for a tissue distribution compartment. Therefore, a two-compartment model with Michaelis-Menten kinetics and linear elimination was used to describe the nonlinear pharmacokinetics of rHuEPO in rats. The parameters obtained by the simultaneous fitting are listed in Table 2. The Michaelis-Menten kinetic parameters were estimated as \(K_m = 67.28 \text{mIU/ml}\) and \(V_{max} = 1993 \text{mIU/h/kg}\). A simpler model with \(k_α = 0\) was tried, but an addition of the linear component of elimination significantly improved the overall fit and reduced the value of the Akaake information criterion (772 versus 720). The nonsaturable elimination \(k_α = 0.209 \text{h}^{-1}\) represents ~30% of overall elimination of rHuEPO at a low-dose linear condition (e.g., \(V_{max}/K_m + k_α \times V_p\)).

The slow absorption process producing \(T_{max} \approx 8\) to 12 h and more prolonged terminal phases were observed in rats after s.c. administration. As seen previously in monkeys (Ramakrishnan et al., 2003) and humans (Ramakrishnan et al., 2004), the characterization of s.c. absorption required a dual
absorption process to account for a fast rising and flip-flop kinetic nature of the data that was not fitted by a single first-order absorption rate constant. The kinetic model suggested a rapid zero-order absorption of part of the s.c. dose absorbed (68%) up to 13.5 h followed by a slow first-order entry from the s.c. injection site. The proposed PK model well described the nonlinear disposition of rHuEPO and s.c. absorption kinetic profiles at different dose levels in rats.

Hematological Changes in Baselines. Notable changes in RBC, Hb, and MCH levels in placebo groups (i.v. placebo and s.c. placebo) were observed during the study period (i.e., the first 4 weeks after dosing), and the negative control group clearly showed time-dependent changes in these variables up to 24 weeks. The baseline patterns observed in all control groups and predicted from the various functions are shown in Fig. 3. The estimated parameters related to the baselines are summarized in Table 3. The gradual increase followed by a plateau in RBC counts was well captured by the Gompertz equation (eq. 9). The value of the growth rate constant \((k_g) = 0.000258 \text{ h}^{-1}\) indicates that the time frame for the initial first-order increase is \(\sim 112\) days, whereas the Gompertz function estimates the true steady-state RBC count of \(785 \times 10^4\) cells/\(\mu\)l reached at \(\sim 258\) days.

The amount of Hb per cell (i.e., MCH) was shown to decline gradually from the initial value, and this decline was reasonably well described by the indirect response model with a time-dependent decrease in the production process \([k_{syn}(t)]\) modeled by a first-order rate constant \((k_d)\). The estimated values of \(k_{syn}\) (0.0888 pg/cell/h) and \(k_{syn-min}\) (0.0769 pg/cell/h) suggested that the production rate of Hb per cell is reduced by 13.4% as rats grow. According to eq. 8, the initial Hb level (Hb\(_0\)) was calculated to be 12.26 g/dl, and there was good agreement between the predicted and observed Hb levels (Fig. 3). Although RET counts showed a slight decrease, overall changes during the study period were modest relative to the treatment effects and thus were handled as a constant baseline from the average value of measurements in the placebo groups.

Pharmacodynamics. The erythropoietic stimulatory effects of rHuEPO administration in rats are shown in Figs. 4 through 7. The data are expressed as means (± S.D.), and the small S.D. of individual data points with great consistency of the data further justified our animal model. Despite the fact that the s.c. administration of rHuEPO produced lower drug concentrations due to incomplete bioavailability, because of prolonged exposure and slow absorption, it produced equal or greater efficacy in rHuEPO treatment compared with i.v. administration at the same dose. This has been also observed in other studies (Boelaert et al., 1989).

As shown in Fig. 4, the peak RET response occurred at \(\sim 4\) to 5 days after the treatments. The dose-dependent increases in RET followed by an immediate decline below the baseline were observed at all dose levels, reflecting the tolerance and rebound phenomena. Then the RET returned slowly to the baseline by days 21 to 24. The mean RBC count and Hb level versus time profiles are presented in Figs. 5 and 6. Because the baseline of RBC count and Hb level increased with time, the observed values for Hb and RBC include treatment effects by rHuEPO as well as the natural incline from the baseline. The RBC responses almost paralleled the Hb profiles. The RBC count and Hb level started steadily rising until peaks were achieved at approximately days 5 to 6 at which point RETs started decreasing because of their conversion into mature RBCs. The increases in RET counts were responsible for this early stage of rise in RBCs and Hb because a newly released RET from the bone marrow is also a

![Fig. 2. Pharmacokinetic profiles of rHuEPO in rats after single i.v. (top) and s.c. (bottom) doses of 450 (●), 1350 (■), and 4050 IU/kg (▲). Symbols represent means ± S.D., and lines are model predictions.](image-url)

TABLE 1
Noncompartmental parameters for rHuEPO pharmacokinetics

<table>
<thead>
<tr>
<th>Parameter (units)</th>
<th>Definition</th>
<th>i.v. Doses (IU/kg)</th>
<th>s.c. Doses (IU/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC(_0-\infty) (IU/ml · h)</td>
<td>Area under the curve</td>
<td>28.07 99.67 312.34</td>
<td>15.51 52.95 185.95</td>
</tr>
<tr>
<td>CL or CL/F (ml/h/kg)</td>
<td>Apparent total clearance</td>
<td>16.03 13.54 12.97</td>
<td>29.01 25.50 21.90</td>
</tr>
<tr>
<td>(k_g) (h(^{-1}))</td>
<td>Terminal slope</td>
<td>0.111 0.0831 0.0786</td>
<td>0.116 0.101 0.088</td>
</tr>
<tr>
<td>(V_{ss}) (ml/kg)</td>
<td>Steady-state volume of distribution</td>
<td>118 115 117</td>
<td>742 2321 8255</td>
</tr>
<tr>
<td>(C_{max}) (mIU/ml)</td>
<td>Maximal concentration</td>
<td>8 12 12</td>
<td>8 12 12</td>
</tr>
<tr>
<td>(T_{max}) (h)</td>
<td>Time that (C_{max}) occurs</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
part of the total number of RBCs measured in blood. Then RBCs gradually declined as the numbers of RETs available for further maturation into RBCs are far below than their usual baseline level due to the tolerance effect, and these phenomena were more marked at the higher dose of rHuEPO.

The MCH levels versus time profiles from control and treatment groups are shown in Fig. 7. Compared with the control groups, administration of rHuEPO yielded slight increases in MCH values until days 4 to 6 followed by slow returns toward normal levels by the end of the study period. Thus, it was assumed that rHuEPO enhances Hb synthesis in cells making Hb, which subsequently increases the amount of Hb per cell. Despite the variability of the data the nonlinear stimulatory function (eq. 12) by concentration of rHuEPO appears to reasonably capture the time course of MCH changes.

The catenary lifespan model with feedback regulatory loop has been used to characterize the erythropoietic effects of rHuEPO (Ramakrishnan et al., 2004). Previously, RET in blood were chosen as a driving force for feedback. However, in this study the Hb change relative to its baseline was considered to be responsible. Adding a Hill coefficient into the stimulatory function did not result in any improvement in model fitting. Parameter estimates obtained by fitting the PD equations to the mean data are listed in Table 4.

$$S_{\text{max}}$$

<table>
<thead>
<tr>
<th>Parameter (units)</th>
<th>Definition</th>
<th>Estimate (CV %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_c$ (ml/kg)</td>
<td>Central volume of distribution</td>
<td>61.18 (3.7)</td>
</tr>
<tr>
<td>$V_{\text{max}}$ (mIU/h/kg)</td>
<td>Michaelis-Menten capacity constant</td>
<td>1993 (—)</td>
</tr>
<tr>
<td>$K_m$ (mIU/ml)</td>
<td>Michaelis-Menten affinity constant</td>
<td>67.28 (28.9)</td>
</tr>
<tr>
<td>$k_{el}$ (h$^{-1}$)</td>
<td>Linear elimination rate constant</td>
<td>0.2209 (4.1)</td>
</tr>
<tr>
<td>$k_{se}$ (h$^{-1}$)</td>
<td>Intercompartment rate constant</td>
<td>0.171 (15.2)</td>
</tr>
<tr>
<td>$k_{sp}$ (h$^{-1}$)</td>
<td>Intercompartment rate constant</td>
<td>0.148 (11.1)</td>
</tr>
<tr>
<td>$k_c$ (h$^{-1}$)</td>
<td>Absorption rate constant</td>
<td>0.146 (24.5)</td>
</tr>
<tr>
<td>$F$</td>
<td>Bioavailability of SC dose</td>
<td>0.586 (3.6)</td>
</tr>
<tr>
<td>$\tau$ (h)</td>
<td>Fraction of dose absorbed by first-order input</td>
<td>0.322 (57.0)</td>
</tr>
<tr>
<td></td>
<td>Time period for zero-order input</td>
<td>13.5 (87.7)</td>
</tr>
</tbody>
</table>

CV, coefficient of variation expressed as S.D./mean.

- Highly variable.

---

TABLE 3
Parameter estimates for hematological baselines

<table>
<thead>
<tr>
<th>Parameter (units)</th>
<th>Definition</th>
<th>Estimate (CV %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_g$ (h$^{-1}$)$^a$</td>
<td>Growth constant in Gompertz equation</td>
<td>0.000258 (4.9)</td>
</tr>
<tr>
<td>$\alpha$ (h$^{-1}$)$^b$</td>
<td>Retarding constant in Gompertz equation</td>
<td>0.00104 (7.5)</td>
</tr>
<tr>
<td>$k_h$ (h$^{-1}$)$^b$</td>
<td>First-order rate constant for time-dependent change in production rate $k_{\text{syn}}(t)$</td>
<td>0.00074 (64.9)</td>
</tr>
<tr>
<td>$k_{\text{syn}}_{\text{min}}$ (pg/cell/h)$^b$</td>
<td>Minimal value of $k_{\text{syn}}(t)$</td>
<td>0.0769 (118)</td>
</tr>
<tr>
<td>$k_{\text{syn}}$ (pg/cell/h)$^b$</td>
<td>Initial value of $k_{\text{syn}}(t)$</td>
<td>0.0888 (119)</td>
</tr>
<tr>
<td>$k_{\text{syn}}_0$ (pg/cell/h)$^b$</td>
<td>Minimal value of $k_{\text{syn}}(t)$</td>
<td>0.0888 (119)</td>
</tr>
<tr>
<td>$k_{\text{out}}$ (h$^{-1}$)$^b$</td>
<td>Loss rate constant</td>
<td>0.261 (15.7)</td>
</tr>
<tr>
<td>$S_{\text{max}}_{\text{MCH}}$ $^b$</td>
<td>Maximal possible stimulation of $k_{\text{syn}}(t)$</td>
<td>87.9 (82.5)</td>
</tr>
<tr>
<td>$SC_{50}$ _MCH (mIU/ml)$^b$</td>
<td>Concentration for 50% stimulation of $k_{\text{syn}}(t)$</td>
<td>612.78$^c$</td>
</tr>
<tr>
<td>$RBC_0$ ($\times 10^6$cells/µl)$^a$</td>
<td>Initial value of RBC count</td>
<td>20$^c$</td>
</tr>
<tr>
<td>$MCH_0$ (pg/cell)$^c$</td>
<td>Initial value of MCH</td>
<td>25.01$^c$</td>
</tr>
<tr>
<td>$RET_0$ ($\times 10^6$cells/µl)$^c$</td>
<td>Initial value of RET count</td>
<td>—</td>
</tr>
</tbody>
</table>

CV, coefficient of variation expressed as S.D./mean.

$^a$ Parameters used for the RBC baseline (eq. 9).

$^b$ Parameters used for the MCH baseline (eq. 10–12).

$^c$ Constants.

---

Fig. 3. Changes in baseline values of RETs (top left), RBCs (top right), mean corpuscular hemoglobin (bottom left), and hemoglobin (bottom right) over time. Symbols depict means ± S.D. from i.v. ( ●), s.c. ( ▲), and negative control groups ( ●), and lines are model predictions. The hemoglobin level was calculated from the RBC count and MCH (eq. 8).

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TABLE 2
Estimated pharmacokinetic parameters of rHuEPO

<table>
<thead>
<tr>
<th>Parameter (units)</th>
<th>Definition</th>
<th>Estimate (CV %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_c$ (ml/kg)</td>
<td>Central volume of distribution</td>
<td>61.18 (3.7)</td>
</tr>
<tr>
<td>$V_{\text{max}}$ (mIU/h/kg)</td>
<td>Michaelis-Menten capacity constant</td>
<td>1993 (—)</td>
</tr>
<tr>
<td>$K_m$ (mIU/ml)</td>
<td>Michaelis-Menten affinity constant</td>
<td>67.28 (28.9)</td>
</tr>
<tr>
<td>$k_{el}$ (h$^{-1}$)</td>
<td>Linear elimination rate constant</td>
<td>0.2209 (4.1)</td>
</tr>
<tr>
<td>$k_{se}$ (h$^{-1}$)</td>
<td>Intercompartment rate constant</td>
<td>0.171 (15.2)</td>
</tr>
<tr>
<td>$k_{sp}$ (h$^{-1}$)</td>
<td>Intercompartment rate constant</td>
<td>0.148 (11.1)</td>
</tr>
<tr>
<td>$k_c$ (h$^{-1}$)</td>
<td>Absorption rate constant</td>
<td>0.146 (24.5)</td>
</tr>
<tr>
<td>$F$</td>
<td>Bioavailability of SC dose</td>
<td>0.586 (3.6)</td>
</tr>
<tr>
<td>$\tau$ (h)</td>
<td>Fraction of dose absorbed by first-order input</td>
<td>0.322 (57.0)</td>
</tr>
<tr>
<td></td>
<td>Time period for zero-order input</td>
<td>13.5 (87.7)</td>
</tr>
</tbody>
</table>

CV, coefficient of variation expressed as S.D./mean.

- Highly variable.
and SC_{50} of rHuEPO in rats were estimated to be 1.87 and 65.37 mIU/ml. The sum of the mean lifespans from cells in bone marrow to RET (e.g., 4.93 days) is very close to the peak time of RET responses, which agrees well with one of typical features observed in the cell lifespan model (Krzyzanski et al., 1999). The mean lifespan of RET (~3 days) obtained from this study represents the average time for RET to be converted into mature RBC in blood once the RET is released from bone marrow, but does not include the maturation time of RET in bone marrow. The feedback inhibition parameter (IC_{50}) was 1.79 g/dl hemoglobin. The PK/PD model well describes RET, RBC, and Hb responses to rHuEPO as well as the tolerance phenomenon after s.c. and i.v. administration. All PD parameters were precisely estimated, and the coefficients of variation were <35% except for T_{p2} (75%).

**Discussion**

This study presents a comprehensive PK/PD analysis of the erythropoietic responses to single i.v. and s.c. administration of rHuEPO in rats at various dose levels.

**Experimental Design.** The accurate estimation of PK parameters requires that multiple samples be taken, often within a short period and PD measurements for rHuEPO require that additional blood sampling be repeated over a period of weeks. These requirements, however, must be balanced against the potential to alter the normal physiological status and to interfere with treatment effects. The rotating sampling strategy used in our study allowed simultaneous determination of PK/PD data without hematological disturbance by bleeding alone. This staggered sampling strategy has been also used successfully for intensive sampling in small animals (Hartley et al., 2003; Jolling et al., 2004) to determine hematological parameters.

**Changes in Hematological Baselines.** Age-related changes in hematological values for various rat strains have been noted (Archer et al., 1982; Turton et al., 1989; Engstrom and Ohlsson, 1990; Kojima et al., 1999). It was reported that the RBC count temporarily decreased within a few days after birth in rats and then increased with age (Kojima et al., 1999). The present study was started at rat age of ~8 weeks, and hematological variables were determined from 8 to 32 weeks of age. A study by Archer et al. (1982) showed that RBC level in healthy Wistar rats reached a plateau at ~18 weeks of age and then stayed constant, which is similar to what we observed. The RBC increase observed in this study was, therefore, thought not to be due to the results of excess blood removal but rather to natural growth of rats. This was confirmed by monitoring the hematological variables in the negative control group over 24 weeks with sparse blood collections. The trends in age-related changes in the hematology of rats were comparable with those seen with inbred strains and other mammals such as rabbits (Laird et al., 1970). These trends were also observed in humans (Bao et al., 1993), and the study reported that Hb levels and RBC counts increased with age for children in the range of 5 to 17 years.

The Hb content per cell was not constant but gradually decreased over time, and this decrease was modeled by a time-dependent decrease in the production rate in the indirect response model. It was assumed that such a decrease is governed by a first-order rate process, but as rats get older...
the production rate reaches a minimal value, and this value is maintained afterward. Then, the stimulation by rHuEPO on the production rate could follow to describe dose-dependent increases in MCH levels by rHuEPO administration. Interestingly, the estimated SC₅₀ for MCH (87.9 mIU/ml) was similar to the SC₅₀ for RBC formation (65.37 mIU/ml). This may suggest that the action of rHuEPO on RBC formation and Hb synthesis in cells resulted from binding of rHuEPO with its receptor that initiates a cascade of intracellular events involved in erythropoiesis.

**Pharmacokinetics.** The proposed PK model well captured the overall profiles of rHuEPO after both routes of administration at each dose level. Nonlinear PK behavior was also observed in the present study, which is consistent with other studies performed in rats (Kinoshita et al., 1992; Kato et al., 1997). Studies have shown that the nonlinear PK of low doses of rHuEPO arises from the saturation of receptor-mediated endocytosis in bone marrow and spleen in humans (Veng-Pedersen et al., 1995) and rats (Kato et al., 1997). A distribution study of radiolabeled rHuEPO in rats revealed that the distribution of rHuEPO in both bone marrow and spleen were saturable at high doses (Kinoshita et al., 1992). However, a saturable distribution was not required in the current model because no trend was observed in the Vₘ₀ with increasing dose. The estimated Kₘ value (67.28 mIU/ml) is almost the same as the SC₅₀ of rHuEPO, suggesting that the receptor binding is closely related to the saturable elimination of the drug (i.e., receptor-mediated drug elimination) (Veng-Pedersen et al., 1999).

The prolonged plasma concentrations after s.c. dosing and slow absorption indicate flip-flop kinetics. The estimated F (58.6%) is in good agreement with the previously determined values of 43 to 62% via noncompartmental analysis after s.c. administration (Kato et al., 2001). No study has previously examined dose-dependent F values of s.c. rHuEPO in rats. Although the dose-dependent F for s.c. dosing was tested and ranged from 0.56 to 0.61, it was not considered significant and, instead, was estimated as a single parameter in the final fitting.

**Pharmacodynamics.** Because of the limit of quantification of the assay, it was assumed that the endogenous EPO is zero in rat plasma. Before rHuEPO administration, EPO receptors are partially occupied by the endogenous EPO that is responsible for maintaining the baseline levels of erythrocytes. The erythropoietic activity of the endogenous EPO in the system is factored into the estimation of the production rate (kₘᵣ) because it is obtained from the baseline and cell lifespan. Moreover, the concentration of rHuEPO exogenously given is significantly larger than endogenous levels in rats. Therefore, any influence of the endogenous level in the current model on the estimation of parameters can be considered to be minimal.

Models that describe pharmacodynamic functional adaptation processes have been introduced on the basis of their primary mechanism (Mager et al., 2003). For the tolerance phenomenon observed in our data, two possible mechanisms relevant to erythropoietic regulation can be considered. First, it could be explained by a negative feedback mechanism driven by
one component (e.g., RETs, RBCs, or Hb) in the circulation. Any changes in these levels will trigger suppression or stimulation of erythropoiesis in the bone marrow to maintain an optimal oxygen supply. This has been postulated by erythropoietin-mediated regulation. The other mechanism could be a precursor pool depletion mechanism. Erythropoiesis consists of a series of cell compartments; one of these cell populations can be depleted by excess stimulation by rHuEPO, and it may take time to fill the depleted cell compartment if the production rate of this precursor is not fast enough. This will subsequently produce tolerance and rebound in the later compartments such as RET and RBC populations.

Negative feedback regulation was proposed to be a primary tolerance mechanism (Ramakrishnan et al., 2004) and, in this present analysis, well describes the PD profiles. Previously, because of a lack of available data (e.g., RBC counts or Hb levels), RETs were necessarily chosen as a regulator of counter-regulation, although Hb was speculated to play a major role in the feedback regulation due to the fact that the oxygen tension in the blood is a primary determinant of erythropoietic control, and Hb is responsible for oxygen delivery (Wintrobe, 2003). The Hb-driven feedback regulation has been adapted by other investigators (Al-Huniti et al., 2004) to model the kinetics and dynamics of EPO.

Although further studies will be needed to confirm which blood component might be involved in the feedback mechanism, it was interesting to compare how the model behaves by each regulator in controlling the timing and degree of the feedback loop. In general, because the RET response is much faster, shorter, and greater than that of Hb, it subsequently introduces a significant oscillating behavior in RET profiles (i.e., quicker recovery to the baseline; data not shown). In contrast, the Hb response would produce a slower and lesser...
feedback loop, resulting in a gradual recovery to the baseline that is more consistent with the observed data. This eliminates the need for the transduction delay parameter (i.e., $T_{PD}$) used in the previous model (Ramakrishnan et al., 2004).

Attempts to describe the data using a combination of the precursor-dependent indirect model with lifespan concept were equally successful in terms of capturing the tolerance phenomenon seen in RET after a single dose (Woo et al., 2004) and multiple dosing regimens (Krzyzanski et al., 2005). However, this model required more parameters to be estimated with less precision. The current model with feedback regulation has fewer numbers of parameters with acceptable precision and thus was finally used to account for the overall PD properties of the system. However, it still remains to be explored experimentally to discriminate which mechanism is responsible for such a complex control process.

In conclusion, a comprehensive mechanism-based PK/PD model well describes the PK profiles and all the PD responses to both i.v. and s.c. dose of rHuEPO in rats. Some PK and PD characteristics of rHuEPO observed in rats were in good agreement with those from other species, and the general PK/PD model structure for rHuEPO applied for other species also holds for rats. The use of rats as an animal model in assessing the PK/PD of rHuEPO should be considered with caution in terms of the total volume of blood removal and the changes in baselines of erythrocyte indices over time. In the present study, the age-related changes in hematological parameters were monitored in rats over a long period of time, and this was taken into consideration in the modeling by a means of various functions. This is particularly important for erythropoietic effects of rHuEPO as well as other drugs that require monitoring of their responses for a long period.

References

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TABLE 4
Estimated pharmacodynamic parameters of rHuEPO

<table>
<thead>
<tr>
<th>Parameter (units)</th>
<th>Definition</th>
<th>Estimate (CV %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$S_{max}$</td>
<td>Maximal possible stimulation of $h_{in}$</td>
<td>1.87 (6.5)</td>
</tr>
<tr>
<td>$SC_{50}$ (mIU/ml)</td>
<td>Concentration for 50% stimulation of $h_{in}$</td>
<td>65.37 (34.6)</td>
</tr>
<tr>
<td>$T_{1/2}$ (h)</td>
<td>Mean lifespan of cells in P1 compartment</td>
<td>42.97 (7.5)</td>
</tr>
<tr>
<td>$T_{RET}$ (h)</td>
<td>Mean lifespan of reticulocytes</td>
<td>3.02 (75.0)</td>
</tr>
<tr>
<td>$IC_{50}$ (g/dl)</td>
<td>Hb required for 50% inhibition of $h_{in}$</td>
<td>72.33 (3.6)</td>
</tr>
<tr>
<td>$h_{in}$ ($10^6$/µl/h)$a$</td>
<td>Zero-order production rate constant</td>
<td>1.79 (9.8)</td>
</tr>
</tbody>
</table>

CV, coefficient of variation expressed as S.D./mean. $a$ Secondary parameter.