An Integrated Pharmacodynamic Analysis of Erythropoietin, Reticulocyte, and Hemoglobin Responses in Acute Anemia

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Purpose. To determine by pharmacodynamic (PD) analysis physiologically relevant parameters of the cellular kinetics of erythropoiesis in acute anemia.

Methods. The PD relationships among erythropoietin (EPO), reticulocyte, and RBC (Hb) responses were investigated in young adult sheep in acute anemia induced twice by two controlled phlebotomies separated by a 4-week recovery period.

Results. The phlebotomies resulted in rapid increases in plasma EPO, with maximal levels occurring at 3 to 8 days, followed by a reticulocyte response with a delay of 0.5 to 1.5 days. The Hb returned to prephlebotomy base line at the end of the 4-week recovery period. The EPO, reticulocyte count, and Hb responses were well described by a PK/PD model ($r = 0.975$) with the following cellular kinetics parameters: the lag time between EPO activation of erythroid progenitor cells and reticulocyte formation; the reticulocyte-to-RBC maturation time; the reticulocyte and Hb formation efficacy coefficients, quantifying EPO's efficacy in stimulating the formation of reticulocytes and Hb, respectively; the C50 PK/PD transduction parameter defined as the EPO level resulting in half the maximum rate of erythropoiesis.

Conclusion. Physiologically relevant cellular kinetics parameters can be obtained by an endogenous PK/PD analysis of phlebotomy data and are useful for elucidating the pathophysiologic etiology of various anemias.

KEY WORDS: Erythropoietin; reticulocytes; hemoglobin; phlebotomy; progenitor cells.

INTRODUCTION

A previous publication (1) examined the pharmacodynamic (PD) relationship between endogenous erythropoietin (EPO) and the reticulocyte response in sheep subjected to phlebotomy. The objective of the present study is to extend the analysis to consider the hemoglobin (Hb) data in the same animals to provide a more comprehensive and endpoint-oriented quantitative examination of the kinetics of EPO's PD effect and identify kinetic parameters of importance in EPO's use.

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MATERIAL AND METHODS

Study animals and study protocol are as previously reported (1).

Laboratory Analysis

Plasma EPO concentrations and reticulocyte counts were determined as previously published (1). Hemoglobin was measured spectrophotometrically using an IL482 CO-oximeter (Instrumentation Laboratories, Watham, MA).

PK/PD Modeling

The kinetic relationship between the EPO plasma concentration profile, $C_{EPO}(t)$, and the relative reticulocyte count, RRC(t), used in the present analysis is based on convolution principles as previously discussed (1), resulting in the following equation derived from Eq. (6) of ref. 1:

$$\begin{aligned} RRC(t) = RRC_{BG}^{SS} \cdot [T_2 - (t - T_1)_+]_+/T_2 \\ + \int_0^t R\{C_{EPO}[(t - T_1)_+]\}\, dt \\ - \int_0^t R\{C_{EPO}[(t - [T_1 - T_2])_+]\}\, dt \end{aligned} \quad (1)$$

In Eq. (1), RRC_{BG}^{SS} is the steady-state background relative reticulocyte count. The rate of erythroid progenitor cells activation is denoted by R, which depends on the EPO concentration, C_{EPO}. The lag time for the formation of the reticulocytes formed from the EPO activation of the erythroid progenitor cells is denoted T_1, and the time it takes a newly formed reticulocyte to mature to a red blood cell (RBC) is denoted T_2. The parentheses in Eq. (1) with the + subscript denote the truncation function, defined as follows:

$$(x)_+ = \begin{cases} x \text{ for } x > 0 \\ 0 \text{ otherwise} \end{cases} \quad (2)$$

The Hb blood concentration is derived similarly to the reticulocyte concentration, resulting in the following equation for the Hb concentration:

$$\begin{aligned} Hb(t) = Hb_{BG}^{SS} \cdot [T_3 - (t - T_1 - T_2)_+]_+/T_3 \\ + K_H\left(\int_0^t R\{C_{EPO}[(t - T_1 - T_2)_+]\}dt \right. \\ \left. - \int_0^t R\{C_{EPO}[(t - T_1 - T_2 - T_3)_+]\}dt \right) \end{aligned} \quad (3)$$

In Eq. (3), Hb_{BG}^{SS} is the steady-state background Hb concentration, T_3 is the life span of the RBCs, and the unit conversion from reticulocyte to RBC is given by the parameter K_H. The progenitor activation rate R in Eqs. (1) and (3) was modeled using a regular E_{MAX} model given by:

$$R[C_{EPO}(t)] = \frac{E_{MAX} C_{EPO}(t)}{C_{50} + C_{EPO}(t)} \quad (4)$$

In Eq. (4), E_{MAX} is the maximum achievable progenitor cell

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activation rate, and C_{50} is the plasma EPO concentration resulting in half the maximum of this rate.

Data Analysis

Equations (1) and (3) were simultaneously fitted to the reticulocyte and Hb data, respectively, using a Windows version of the general nonlinear regression program *FUNFIT* (7). The baseline reticulocyte, Hb, and plasma EPO measurements were used as fixed parameters. This was done by averaging the two or three time points obtained before the onset of the phlebotomy procedure. The plasma EPO concentration $C_{EPO}(t)$ was represented using a generalized cross-validated spline function (8). Comparison to previous results was done with a paired *t*-test. Statistical tests were performed using SAS/STAT (SAS Institute Inc., Cary, NC).

RESULTS

The reticulocyte, EPO, and Hb data have previously been summarized (1). The extended model [Eqs. (1) and (3)] in the current study, which was fitted simultaneously to the reticulocyte and Hb data, showed excellent agreement with the observed data ($r = 0.975$, Table I). Figure 1 provides representative plots of such fittings. Table I summarizes the parameter estimates and also includes for comparison previously reported estimates for the T_1 and T_2 parameters (1). The primary parameters estimated in the fitting of Eqs. (1) and (3) were T_1, T_2, K_H, E_{MAX}, and C_{50}. The RBC life-span parameter, T_3 could not be determined because the length of the experiments was less than the anticipated life span of RBCs. However, this parameter has been accurately determined in sheep using a permanent [^{14}C]cyanate label and was found to be 114 days (9). Accordingly, T_3 was set as a fixed, constant parameter of 114 days in the model fittings. Individual estimation of the E_{MAX} and C_{50} parameters could not be determined in two of the 10 phlebotomy cases because the PK–PD transduction function, Eq. (4), was reduced to the following linear relationship as a result of the C_{50} values being large relative to the maximum plasma EPO concentrations:

$$R[C_{EPO}(t)] = \frac{E_{MAX} C_{EPO}(t)}{C_{50} + C_{EPO}(t)} \rightarrow \frac{E_{MAX}}{C_{50}} C_{EPO}(t) \quad \text{for} \quad C_{50} \gg C_{EPO} \tag{5}$$

Table I summarizes the values for the E_{MAX}/C_{50} and $K_H E_{MAX}/C_{50}$ ratios, important in assessing EPO's efficacy in producing reticulocytes and Hb, respectively. The table also summarizes values for C_{MAX}/C_{50} and gives the degree of nonlinearity experienced at the peak EPO level following the phlebotomy. The C_{MAX}/C_{50} ratio exceeds 1 in half of the cases.

DISCUSSION

PK/PD Modeling Rationale

The proposed PK/PD system analysis model consists of two components, both of which make use of convolution principles to model cellular transformation processes. The first component relates the reticulocyte responses to the endogenous plasma EPO concentration, and the second relates the Hb response to the plasma EPO concentration. Details of the first part are described in a previous publication, in which both relative and absolute reticulocyte counts yielded equivalent results (1). In the present work, all the modeling was performed using relative reticulocyte counts.

The reticulocyte count vs. time response, RRC(t), depends on the EPO plasma concentration, C_{EPO}, according to a convolution-type system analysis model (1). The model involves a reticulocyte unit impulse response function (UIR), which defines the fundamental cellular state vs. time transition following cellular activation. The reticulocyte UIR corresponds to the transformation of an erythroid progenitor cell activated by EPO. At a time T_1, measured relative to the time of the activation, the erythroid progenitor cell becomes a reticulocyte available for sampling in the blood stream. It remains identifiable as a reticulocyte for a finite time, T_2, and then becomes a mature RBC at time $T_1 + T_2$ relative to the time of the activation. Thus, for the reticulocyte formation process, UIR = 1 when the activated cell has become a reticulocyte, whereas UIR = 0 signifies that the cell is not a reticulocyte.

The first term of Eq. (1) is the *background* reticulocyte response, defined as the reticulocyte response created by EPO's progenitor activation taking place before the start of the phlebotomy ($t < 0$). The first term accounts for a steady-state background reticulocyte count RRC_{BG}^{SS} present at $t = 0$. This steady-state count is created through a constant activation rate, $R(C_{EPO}^0)$, resulting from an assumed constant EPO baseline level, C_{EPO}^0, present before the start of the phlebotomy ($t < 0$). This background term decays in a linear fashion to zero with a delay = T_1 according to a fractional decay function $[T_2 - (t - T_1)_+]_+/T_2$ [Eq. (1)]. The second term of Eq. (1) represents the "*foreground*" response, defined as the reticulocyte response resulting from EPO's progenitor cell activation taking place after the start of the phlebotomy ($t > 0$). As a simple check, it is readily verified that in the hypothetical case when the EPO concentration is not changing from its

Table I. Summary of PK/PD Parameters Estimated from Eqs. (1) and (3) Simultaneously Fitted to Plasma EPO Concentration, Relative Reticulocyte Count, and Hemoglobin Data

	T_1[a] (days)	T_2[a] (days)	E_{MAX} (%/day)	E_{MAX}/C_{50} (%/day)/(U EPO/ml)	K_H (g Hb/dl)/%	$K_H * E_{MAX}/C_{50}$ [(g Hb/dl)/day]/(U EPO/ml)	C_{MAX} EPO (mU/ml)	C_{50} EPO (mU/ml)	C_{MAX}/C_{50}	r[a]
Mean (n = 10)	0.873/0.47	3.37/4.98	11.5	30.6	0.227	5.68	760	601	3.37	0.975/0.950
SD	0.344/0.28	1.20/1.31	5.32	23.2	0.0695	1.70	403	552	2.90	0.012/0.04
CV %	39.4/59.7	35.6/26.2	46.3	75.9	30.6	30.0	53.1	91.8	86.2	1.23/4.5

[a] The second value is from the previous study (1), which does not include Hb in the PK estimation.

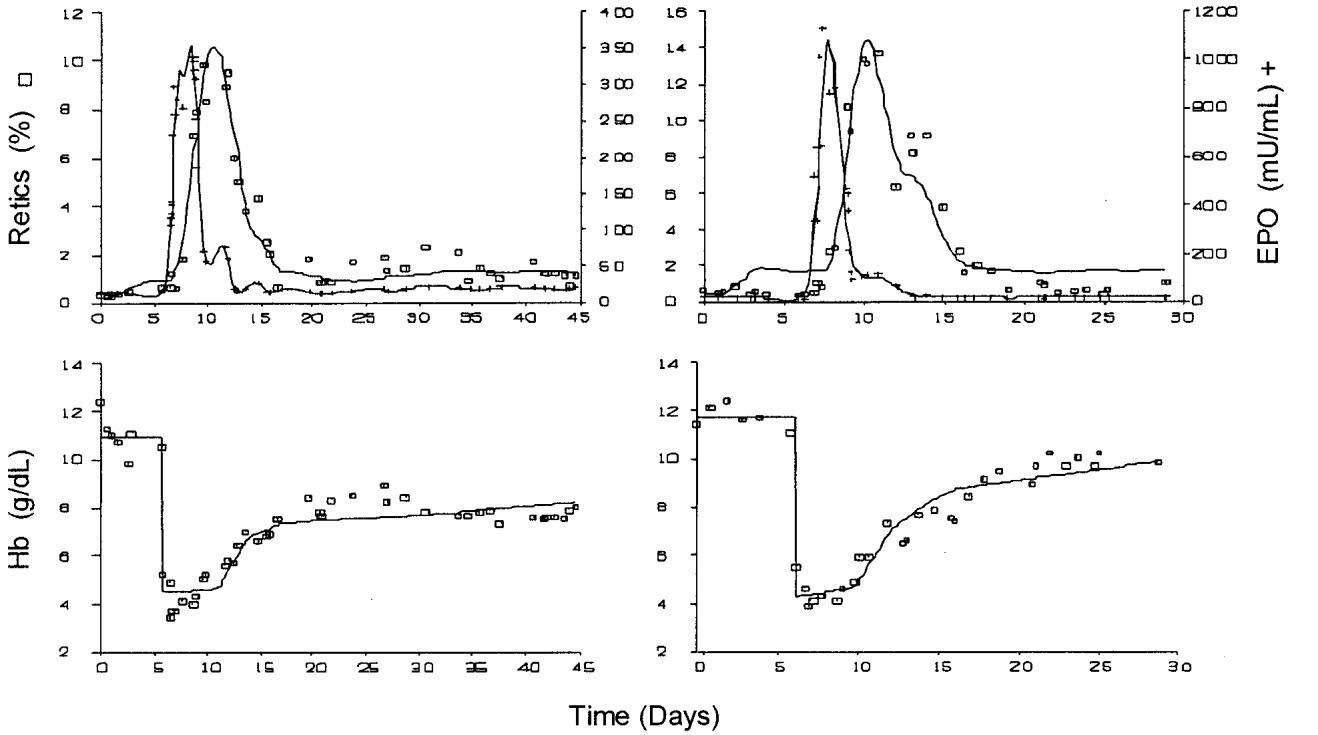

Fig. 1. Two representative plots of the model [Eqs. (1) and (3)] simultaneously fitted to EPO, reticulocyte, and hemoglobin data.

baseline value, RRC will remain constant, equal to RRC_{BG}^{SS} as expected. This situation corresponds to a delayed (delay = T_1) "foreground" reticulocyte response equal to $RRC_{BG}^{SS} \cdot \{T_2 - (T_2 - (t - T_1)_+]_+\}/T_2$. In this case RRC remains constant consistent with the fact that the sum of the background and foreground responses is equal to RRC_{BG}^{SS}; i.e.,

$$RRC = RRC_{BG}^{SS} \cdot [T_2 - (t - T_1)_+]_+/T_2 + RRC_{BG}^{SS} \cdot \{T_2 - [T_2 - (t - T_1)_+]_+\}/T_2 = RRC_{BG}^{SS} \quad (6)$$

(A similar check verifies that Hb = constant = Hb_{BG}^{SS} under these conditions). Equation (3) relating the Hb response to the EPO plasma concentration is based on similar principles applied to model the reticulocyte response. Equations (1) and (3) differ only with respect to the UIRs and the use of the conversion constant K_H in Eq. (3). The UIR for RBC (Hb) formation is given by:

$$UIR_{RBC}(t) = \begin{cases} 1 \text{ for } T_1 + T_2 < t < T_1 + T_2 + T_3 \\ 0 \qquad\qquad \text{otherwise} \end{cases} \quad (7)$$

It shares the same parameters, T_1 and T_2, used in the reticulocyte UIR. This is because the time, $T_1 + T_2$, when a reticulocyte becomes a RBC coincides with the time when the RBC is created. The additional UIR parameter T_3 of RBC [Eq. (7)] defines the life span of the RBCs. The conversion constant K_H in Eq. (3) does the unit conversion from the reticulocyte count to the corresponding Hb level in the transformation from reticulocytes to RBCs to Hb. The discontinuous nature of the UIRs is accounted for by the "truncation function," Eq. (2), applied in Eqs. (1) and (3).

E_{MAX}, C_{50}, and the E_{MAX}/C_{50} Ratio

The analysis makes use of a simple E_{MAX} model [Eq. (4)] for the PK/PD transduction function, which has shown great utility in many PK/PD analysis situations. The E_{MAX} model usually enables the determination of the maximum achievable rate, E_{MAX}, to be estimated in addition to the nonlinearity parameter C_{50}, which defines the concentration resulting in half the maximum achievable rate. However, the estimation of these parameters becomes troublesome in cases with C_{50} values that are large relative to the EPO concentration range used in the determination, or equivalently, in cases in which concentrations are small relative to the value of the C_{50}. This estimation problem was encountered in two cases in this study, which required a fitting using a linear, rather than a nonlinear, PK–PD transduction function [Eq. (5)]. Thus, in these cases the ratio E_{MAX}/C_{50} was estimated as a primary fitting parameter rather than calculated from individually estimated E_{MAX} and C_{50} parameters, as was done in the other cases. The cause of this estimation "problem" was not the particularly low EPO concentrations because the C_{MAX} values for these two linear cases were not substantially below the other C_{MAX} values. Instead, the PK/PD transduction linearity appears to be caused by comparatively much larger C_{50} values in these two cases.

The inability to reliably estimate E_{MAX} and C_{50} individually in these two cases was not a real problem because in both the linear and the nonlinear cases, the E_{MAX}/C_{50} ratio can readily be determined. This ratio is particularly useful because it provides a measure of the efficacy of EPO in stimulating the production of reticulocytes. The units for this ratio is (%/day) per (U/ml), corresponding to a rate of reticulocyte formation (%/day) relative to the EPO concentration (U/ml). This production rate/concentration ratio will be constant and equal to E_{MAX}/C_{50} as long as EPO is operating in the linear PK/PD transduction concentration range, i.e., at an EPO concentration substantially lower than C_{50}. Thus, to compare the linear and nonlinear transduction cases, an extrapolation of

the EPO concentration down to the linear range (e.g., $C_{EPO} < 0.05C_{50}$) is required for the nonlinear case for a direct comparison via the E_{MAX}/C_{50} ratio. The EPO baseline plasma levels were all in the linear range. Accordingly, the E_{MAX}/C_{50} ratio (Table I) becomes a first-order activation rate constant at the baseline and in the linear range ($C_{EPO} < 0.05C_{50}$).

The mean value of the ratio C_{MAX}/C_{50} of 3.37 ± 2.90 indicates for the nonlinear cases that the EPO plasma concentrations reached into the nonlinear concentration range, corresponding to a nonlinear transduction. The fact that the C_{MAX}/C_{50} ratio exceeds 1 in half of the cases indicates that a significant degree of nonlinearity is present. Simplifying such cases with a linear transduction function may introduce errors in the transduction rate of the order of 200% or more [Eqs. (4) and (5)]. Not surprisingly, in the majority of the cases (8 out of 10), a nonlinear transduction function was the preferred model as judged according to the Akaike information criteria (23).

The maximum plasma EPO level reached in humans by clinical subcutaneous injections of 50 U/kg is of the order of 40 mU/ml (10). This is less than 10% of the mean C_{50} value as determined by the present work. This corresponds to only a slight degree of nonlinearity at the peak levels. However, if the same dose were injected as an intravenous bolus dose, then much larger C_{MAX} values would be encountered, reaching well into the nonlinear transduction range. The difference between the maximum plasma EPO concentration in i.v. bolus vs. subcutaneous dosing is further amplified by the complete bioavailability in the i.v. bolus case compared to the reduced bioavailability reported in subcutaneous injections (10).

Transduction Nonlinearity, EPO Efficacy, and Mode of Administration

EPO's efficacy is determined kinetically by three parameters, namely, the transduction nonlinearity as quantified by the C_{50} parameter; the E_{MAX}/C_{50} ratio; and $K_H E_{MAX}/C_{50}$. The main implication of transduction nonlinearity is the effect it has on the efficacy of the drug. Drug level profiles in the nonlinear range (concentrations close to or above the C_{50} value) are less efficacious than drug levels in the linear range when compared on an equal-dose basis. This finding is consistent with findings in human studies, where subcutaneous administration of EPO was found to be more efficacious than the same dose given intravenously (10,12). Because the linear range in humans is unclear, studies are needed to evaluate C_{50} and establish the linear range for plasma EPO in humans. A 100 U/kg intravenous bolus dose of EPO in humans results in a peak level as high as 2000 mU/ml. Thus, if the C_{50} value (Table I) determined in this study is comparable to that of rhEPO in humans, such an intravenous administration of rhEPO would drive the plasma concentration well into a very nonlinear PK/PD transduction range, which would reduce EPO's efficacy because EPO's PD response relative to its concentration diminishes at higher concentrations in the nonlinear concentration range.

Accordingly, the relatively slow release from a subcutaneous injection is advantageous in producing a more protracted and lower drug level. However, subcutaneous injections are reported to have a reduced bioavailability, which will offset to some extent the advantage from the lower drug level profile. Subcutaneous bioavailability of rhEPO in humans varies significantly between 10% and 50% among studies regardless of doses (11–14). After subcutaneous EPO administration, EPO plasma concentration is also widely variable between 8 and 30 h, indicating a variable release mechanism from subcutaneous injection sites. This could also be caused by a variable "vascular overflow." The latter process is characterized by a variable fraction of the drug getting into the general systemic circulation at the time of the injection and not being deposited as a subcutaneous slowly released depot. The rate of absorption depends on the site of subcutaneous administration, but there is apparently no correlation between the rate of absorption and bioavailability.

Hemoglobin Production and the Hemoglobin Formation Efficacy Coefficient

The values determined for HFEC = $K_H E_{MAX}/C_{50}$ presented in Table I quantify the efficacy of EPO in its production of Hb. HFEC indicates the Hb formation rate relative to the EPO concentration. The units for HFEC are Hb formation rate, in grams of Hb per deciliter of whole blood per day, relative to the EPO plasma concentration measured in units of EPO per milliliter. The ratio between the Hb production rate and the EPO plasma concentration is constant and equal to HFEC when the EPO concentration is in the linear PK/PD transduction range (e.g., $C_{EPO} < 0.05C_{50}$). All baseline EPO levels were in the linear range; thus, the HFEC parameter, which is a linear-range, first-order rate parameter [Eq. (10)] is descriptive of the baseline-level and linear-range EPO efficacy for Hb production. HFEC appears well determined as judged by the small intersubject variability (30% CV). HFEC demonstrated the smallest intersubject variability of all the parameters determined (Table I). Accordingly, the determination of HFEC ($K_H E_{MAX}/C_{50}$) under different pathologic or physiologic conditions may provide a precise measure of the erythropoiesis that may help expand our knowledge and understanding of important factors in erythropoiesis.

Recovery of Hb

The rate of Hb recovery during the first 10 days postphlebotomy in the sheep of this study is remarkably fast compared to the Hb gain in chronic rhEPO therapy in human renal failure patients. In this study, the mean Hb level 10 days after the phlebotomy had recovered to 7.6 ± 0.7 g/dL. In humans, a period of 2 to 4 months is often required to achieve a significant increase, e.g., 2–3 g/dL, in Hb level in rhEPO therapy in chronic renal patients or patients with anemias from other origins (15). There may be several reasons for the marked difference in PD effect of EPO: endogenous EPO might differ from rhEPO in terms of its efficacy per unit dose, or other growth factors may be induced to a larger extent under the more stressed condition (Hb ~4 g/dL), e.g., insulin-like growth factor-1 or "hypoxia-inducible factor" (HIF-1) (16,17). It could also be related to species difference. Our observations also suggest that there may exist endogenous compounds released in response to hypoxia with significant synergy to recombinant EPO. Identifying such endogenous substances opens up the possibility of developing combina-

tion therapies and new treatment strategies for treating anemia more effectively.

The T_1 and T_2 Parameters

The mean values for the T_1 and T_2 parameters estimated in this work are 0.873 and 3.37 days, respectively (Table I), which are comparable to but statistically significantly different ($p < 0.05$) to the estimate (0.47 and 4.98 days) from the previous work based on reticulocyte data alone (1). The current estimates should be more accurate because the parameters are based on a more comprehensive analysis involving both reticulocyte and Hb data. The use of a nonlinear transduction function, which led to significantly better fits as judged by the correlation coefficients ($p < 0.05$, Table I), may also be a contributing factor to a better estimation. The T_2 parameter represents the average reticulocyte maturation time, and its estimate under the phlebotomy-stimulated condition is larger than the maturation time under nonstimulated, normal conditions, which has been determined to be 1–2 days (18–20). Our larger T_2 values suggests that immature reticulocytes produced under stressed conditions require a longer time to mature into RBCs.

Second Phlebotomy vs. First Phlebotomy

Because each of the sheep in the present study was phlebotomized twice, it was of interest to test whether there was a systematic change in the parameter estimates between the first and second phlebotomy periods, but no time-dependence was found in the paired comparisons of individuals ($p > 0.05$). It is surprising to note that one subject showed a linear PK/PD transduction in the first phlebotomy but a nonlinear transduction in the second. Another subject showed the same difference, but in a different order, which indicates that the apparent shift from a predominantly linear to nonlinear transduction is not dependent on the phlebotomy but on other factors. However, these differences may not represent a fundamental change in the PK/PD transduction mechanism but rather may signify a substantial change in the C_{50} parameter. The reasons for such a change is not clear. The fact that none of the study animals showed significant changes in plasma iron concentration or in the plasma iron TIBC saturation (1) indicates that sufficient iron was available for the erythropoiesis. This is also consistent with the lack of significant difference observed in the kinetics between the first and second phlebotomy.

In summary, this work presents a pharmacodynamic analysis of EPO's erythropoietic effect based on a "natural" physiologic experiment in which erythropoietic variables and derived PK/PD parameters are observed in response to phlebotomy-induced anemia. Novel PD parameters quantifying the erythropoietic efficacy of EPO have been presented, i.e., the reticulocyte formation efficacy coefficient, and the hemoglobin formation efficacy coefficient, in addition to the time parameters T_1 and T_2 for the cellular transformations from erythroid progenitor to reticulocyte to RBC. These parameters provide new insight into erythropoiesis and establishes an expanded foundation for development of better dosing/treatment strategies with EPO. The proposed kinetic PK/PD model enables an in-depth analysis of the erythropoiesis, useful for identifying and understanding how different biologic factors, e.g., cytokines, and different pathophysiologic conditions might influence the erythropoiesis.

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