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*Brief/Technical Note*

# Overlooked Issues on Pharmacokinetics Data Interpretation of Protein Drugs—a Case Example of Erythropoietin

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Recombinant human erythropoietin (rhEPO) has been used for over 30 years as a major therapeutic agent for treatment of anemia caused by chronic kidney disease and chemotherapy. During this period, a great deal has been learned about rhEPO and its physiological role in the regulation of erythropoiesis (1). Nevertheless, several issues remain to be resolved in the rhEPO pharmacokinetics arena due to the challenges of unifying different assays and different EPO standards. In this communication, we report two frequently overlooked issues on pharmacokinetics data interpretation of rhEPO and offer solutions to ensure reliable estimation of the pharmacokinetics parameters. To the best of our knowledge, there have been no reports on these important issues.

## BACKGROUND OF EPO MEASUREMENTS

Unlike small-molecule compounds whose amounts are expressed in grams or moles, EPO amounts are expressed in units (U), which is defined based on EPO's biological activity determined by *in vivo* bioassays (2). More specifically, 1 U of EPO is defined as the amount of EPO that stimulates erythropoiesis in rodents equal to that of 5 μM of cobalt. As the unit of EPO dose is assigned based on its potency and not the actual amount of carboprotein, the value can vary due to differences in EPO's specific activities, which are subject to many factors, such as different cell lines used for rhEPO biosynthesis, different purification methods, and different manufacturers (3,4). For example, the specific activities reported for rhEPO range from 100,000 to 200,000 U/mg of protein, with the variability mainly caused by differences in the carbohydrate composition of EPO from various sources (4).

The assays used for quantification of rhEPO in biological samples are mainly immunological assays, which measure the binding of the EPO to anti-EPO antibodies. Early EPO immunoassays used the radioimmunoassay format, while nowadays the most common one is the sandwich immunoassay in which the amount of EPO is usually measured by a colorimetric or chemiluminescent reaction (4). The specificity and sensitivity of the immunological assays are dependent on the assay format and on the specificity and affinity of the specific anti-EPO antibodies employed. In most EPO assays, the EPO concentration is measured by comparing EPO standards, which are calibrated against reference preparations, in particular the Second International Reference Preparation (2nd IRP) of urinary EPO and the newer International Standard (IS) for rhEPO (4). The IS for rhEPO includes the first International Standard (1st IS, NIBSC code 87/684), the 2nd IS (code 88/574), the 3rd IS (code 11/170), and two other candidate ISs (codes 87/690 and 87/696) (3,5). The bulk rhEPO used in preparing the 1st IS and 2nd IS were synthesized in Chinese hamster ovary cell lines by different manufacturers (6). The remaining ISs were synthesized in baby hamster kidney cell lines (87/690 and 11/170) and mouse C127 fibroblast cell lines (87/696), respectively (5,6).

## OUR OWN EXPERIENCE—TWO OVERLOOKED ISSUES ON PHARMACOKINETICS DATA INTERPRETATION OF RHEPO

Based on the above background information, it is evident that EPO measurements should be evaluated with the utmost care as discrepancies between expected and measured results may occur if comparisons are made between EPO preparations of different origin or between results of different types of EPO assays. The objective of this communication is to discuss such problems that we have encountered in pharmacokinetics data interpretation of rhEPO in our

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**Table I.** rhEPO Concentration in Two Lots of Dosing Vials Using MSD Immunoassay

| | Plate 29<br>Mean (CV%), U/mL | Plate 30<br>Mean (CV%), U/mL | Plate 31<br>Mean (CV%), U/mL |
|---|---|---|---|
| EPO lot number G28 (3000 U/mL) | 1938 (7%) | 2002 (9%) | 1729 (6%) |
| EPO lot number G27 (3000 U/mL) | 1875 (5%) | 2061 (8%) | 1709 (3%) |

Fresh vials from 2 of the lot numbers of Janssen 3000 U/mL EPO (PROCRIT®) were opened and diluted 1:300 to get a 10,000 mU/mL working stock. Fifty microliters of PROCRIT® up to 500 μL (1:10) then 50 μL 1:10 up to 1500 μL (1:30) for an overall 1:300 dilution at 10 U/mL or 10,000 mU/mL (i.e., the same EPO concentration as the MSD Hypoxia Kit EPO Standard before the 1:4 serial dilutions used for the Standard Curve). These working stocks were similarly diluted 1:4 (50 μL up to 200 μL) and analyzed in 3 successive Hypoxia Plates (29, 30, and 31). $N = 5$ within each plate and experiment was repeated three times (plate 29, plate 30, and plate 31)

EPO human pharmacokinetic studies, and offer solutions to these problems.

### Issue 1: in a Pharmacokinetic Study—Pharmacokinetic Parameter Estimation Can Be Inaccurate Without Checking the Amount of EPO in the Dosing Vial

We noticed this issue in our recent EPO pharmacokinetic study conducted in preterm infants (data not published). In this study, each infant received up to 10 rhEPO doses (600 U/kg or 1200 U/kg) within 4 weeks and EPO plasma samples were collected at various time points. The rhEPO used in the study (PROCRIT®) was provided by Janssen Pharmaceuticals. We measured the concentration of rhEPO in plasma samples using a commercial sandwich chemiluminescence assay, which was developed and validated by Meso Scale Discovery (MSD, Rockville, MD). The EPO standards provided in the assay kits were calibrated against the 2nd IS (code 88/574). In addition to rhEPO plasma samples, we also measured the rhEPO concentration in two different lots of rhEPO dosing vials used in the study. In both lots, we observed that the *apparent* rhEPO dose detected using our assay was approximately 60% of the labeled rhEPO dose with small lot-to-lot variability (Table I). This is not surprising since Janssen may have used EPO reference preparations of different origin and/or different EPO assay. As we were unable to obtain proprietary assay information or the reference standard used for determining EPO concentration in their dosing vials, it is difficult to identify the exact factor(s) that caused this discrepancy. One possibility is that a different rhEPO IS may have been used by Janssen to calibrate their in-house standards. We compared the signal of the EPO standards provided in the MSD assay kits with that from 1st IS and 3rd IS. As shown in Fig. 1, within the same plate, the signals of the standards prepared using the 3rd IS were clearly lower than the EPO standards used in our assay (which had been calibrated against the 2nd IS). On the other hand, the signals of the standards prepared using the 1st IS were close to those from the MSD's EPO standards. As noted earlier, both 1st IS and 2nd IS were synthesized in Chinese hamster ovary cell lines, but the 3rd IS was synthesized in baby hamster kidney cell line. The different cell origin may have caused this discrepancy between the 3rd IS and MSD's EPO standards.

Regardless of the reason that caused this discrepancy, from a pharmacokinetic perspective the more important and relevant question is "what can we do to ensure an accurate EPO pharmacokinetic parameter estimation?" Our recommendations are the following: (1) EPO concentrations in the dosing vials should be measured using the same assay that is used for analyzing the EPO plasma samples, and (2) an EPO assay calibration factor should be applied to calculate an apparent dose for use in determining pharmacokinetic parameters. In our case, the correction factor was 0.6. It is worth emphasizing that the estimated pharmacokinetic parameters (such as CL and Vd) will not be accurate unless the

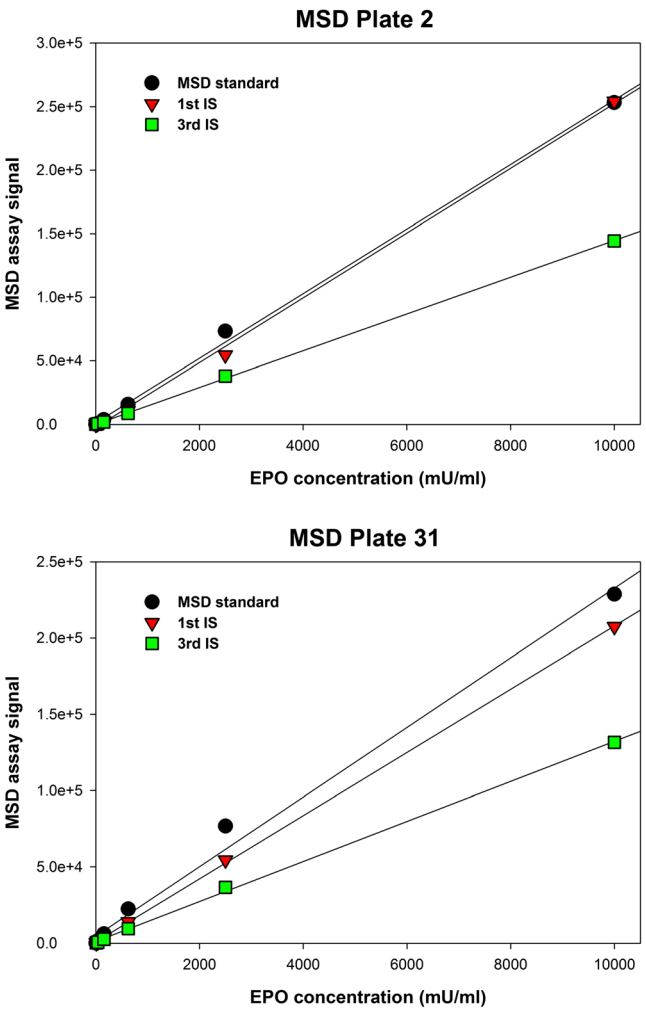


**Fig. 1.** The assay signals of the MSD standards, 1st IS, and 3rd IS of EPO within the same plate (top: MSD plate 2; bottom: MSD plate 31). Each sample was assayed twice within each plate and experiment was repeated using two plates (MSD plate 2 and MSD plate 31)

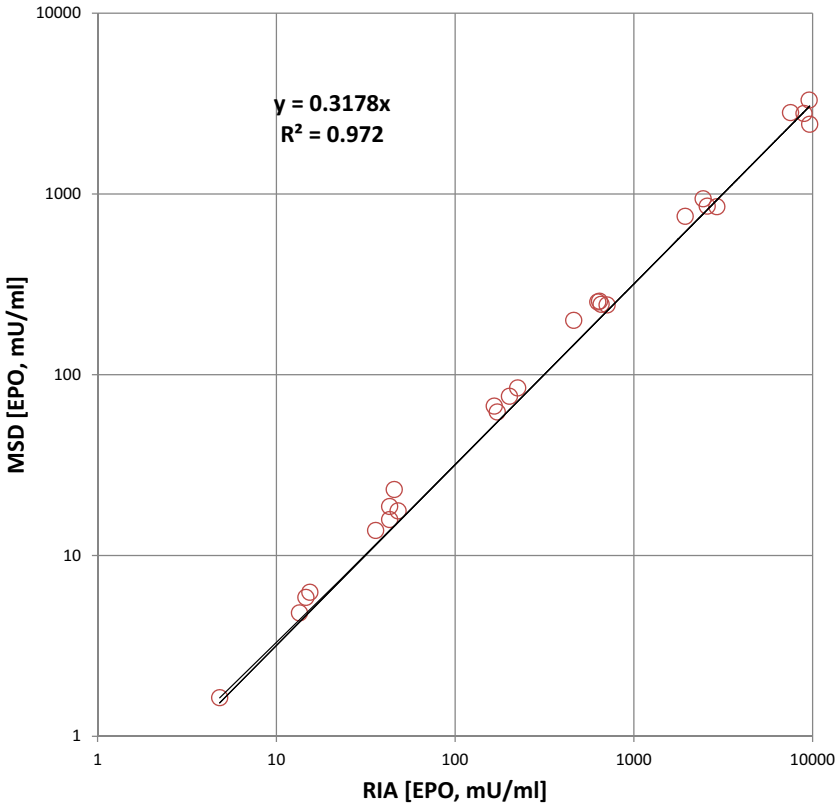


**Fig. 2.** Comparison of EPO concentrations (mU/mL) measured using MSD method (*y*-axis) *vs* RIA method (*x*-axis). Each sample was measured twice. *T* test was performed on the slop coefficient; $p < 0.001$. MSD method: the plasma concentration of Epo was determined using a commercial electrochemiluminescence detection plate assay (Human Hypoxia Serum/Plasma Kit no. K15122C, Meso Scale Discovery, Rockville, MD) following the manufacturer's recommended procedure. The assay covered an expansive dynamic range (2.4 to 10,000 mU/mL) with an LLOQ of 2.0 mU/mL. Inter-assay variation % CV and intra-assay % CV averaged 14.0% and 4.6%, respectively. RIA method: the plasma concentration of Epo was measured using a double-antibody radioimmunoassay (7,9). The linear range of the assay was 4–62.5 mU/mL. The LLOQ of the assay is 1.15 mU/mL. Inter-assay variation % CV and intra-assay % CV averaged 6.4% and 3.5%, respectively

input (i.e., dose) and output (i.e., concentration) are measured using the same assay system. Therefore, it is important and necessary to make sure that *both* EPO concentrations in the dosing vials and plasma samples are evaluated by *the same method and the same reference standard*. We noticed that most research groups which have worked on EPO pharmacokinetics studies only measured EPO concentrations in plasma/serum samples without confirming if the labeled dose still remains at the same value using their own assay. We hope this brief/technical note can serve as a guide to facilitate the best practice in future EPO clinical pharmacokinetic studies.

### Issue 2: a Direct Comparison of EPO Pharmacokinetic Exposure (e.g., AUC and Cmax) Across Different Pharmacokinetic Studies May Be Inappropriate if Different Types of Assays Are Used

As noted earlier, the EPO assay used in our current pharmacokinetic study was a sandwich chemiluminescence assay (data not published). Many years ago, we conducted two EPO clinical pharmacokinetic studies in different populations in which the rhEPO samples were measured using a well-validated radioimmunoassay in which 1st IS was used as the calibration standard (7,8). Since different EPO assays were used, a natural question arose about the appropriateness of comparing the EPO pharmacokinetic exposures among these different studies. With this question in mind, we conducted an exploratory study in which 26 human EPO samples with concentrations ranging from low to high were analyzed using both a radioimmunoassay (RIA method) and a chemiluminescence assay (MSD method). As shown in Fig. 2, the EPO concentrations measured using the chemiluminescence assay were substantially lower than those measured using the radioimmunoassay. This suggested that it is inappropriate to make direct comparison of the EPO pharmacokinetic exposure obtained from our recent EPO study with that from those two earlier pharmacokinetics studies. However, although there was a large discrepancy for each sample between these two assays, the magnitude of the difference remains consistent across all samples and is concentration-independent, with the values measured using the chemiluminescence assay being around 32% of those

measured using the radioimmunoassay (Fig. 2, slope is 0.3178). This linear relationship indicated that performing EPO pharmacokinetic exposure comparison among different studies remains possible if an assay calibration factor (i.e., concentration correction factor in this case) is implemented.

## CAN THE EXPERIENCES LEARNED FROM EPO BE EXTRAPOLATED TO OTHER PROTEIN DRUGS?

As with EPO, several other protein drugs, such as insulin and somatropin, are also expressed in units which are defined based on their biological activity. In this case, both issue 1 and issue 2 that we observed in EPO may be present for these protein drugs as well. On the other hand, issue 1 observed in EPO may not be a problem for antibody drugs, as their dose is expressed in amounts, not units. Because the methodology used for quantification of protein drug primarily includes immunological assays, issue 2 observed in EPO may also exist with other protein drugs, including antibody drugs, as it is not uncommon to switch assays during the clinical drug development. Although the concentration correction factor for issue 2 was found to be concentration-independent for EPO, it may not hold true in all cases. Nonlinear relationship (i.e., concentration-dependent) could happen depending on the complexity of the assays and the nature of the protein products. This is particularly true for monoclonal antibody drugs (mAb), where multiple forms of mAb and ligands (L) exist, including free mAb, free L, and complexes of mAb and L. Due to the complexity of the dynamic binding equilibrium occurring for mAb, any differences in the assay methodologies, such as differences in dilution, incubation times, or potency of the capture and detection reagents, potentially could impact the PK/PD measurement (10). In this case, PK/PD modeling and simulation may serve as a valuable tool to characterize the nonlinear relationship and correct the measurements accordingly.

This brief note will be particularly useful to those researchers who work on pharmacokinetics studies of protein products but are less experienced with the bioanalysis of those drugs. In addition, this brief note may also benefit those pharmacokinetics experts whose expertise is mainly on small-molecule drugs and who are less familiar with these issues as the situation is unique for protein drugs, especially those protein drugs whose dose is assigned based on potency. On the other hand, the issues mentioned in this brief note will not apply to the biosimilar studies because of the head to head pharmacokinetics comparison between the biosimilar product and the originally approved product within the same study (the assay and the standard are unified in that situation). Similarly, the issues we pointed out may not be an issue for a protein drug candidate being developed by a pharmaceutical company intending to use same validated assays for all clinical trials with the drug candidate.

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## COMPLIANCE WITH ETHICAL STANDARDS

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