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Development and validation of a quantitative cell-based bioassay for comparing the pharmacokinetic profiles of two recombinant erythropoietic proteins in serum

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Abstract

An *in vitro* cell-based bioassay was developed and validated to assess the pharmacokinetic profiles of two novel therapeutic recombinant proteins (EP1 and EP2) with erythropoiesis stimulating properties in Sprague–Dawley rats. While immunoassays are the standard choice for evaluating the pharmacokinetic parameters of drugs, no immunoassay was available for EP2, necessitating the need for a quantitative bioassay capable of measuring both EP1 and EP2 separately so that appropriate comparisons could be made. The bioassay described here utilizes a sub clone of the murine 32D cell line transfected with the gene encoding for the human erythopoietin (HuEPO) receptor. Erythropoietin (EPO), EP1 and EP2 exert their proliferative effect on the cell line by signaling through the HuEPO receptor. The proliferation induced by the erythropoietic proteins was measured by [methyl-^{3}H]thymidine incorporation into the cellular DNA. The assay was conducted in 96-well microtiter plates and had relatively high throughput. The Guidelines of the International Conference on Harmonization (ICH) were followed for the validation of the different assay parameters including robustness, linearity, accuracy, precision, limit of quantitation (LOQ) and specificity. The robustness of the bioassay is demonstrated by the lack of an effect of age of the 32D cell culture on the performance of the EP2 bioassay. The bioassay demonstrated good linearity, yielding a coefficient of determination of 0.99 or higher for both EP1 and EP2. The assay showed reproducible dose–response curves for EP1 in the range of 0.039–2.5 ng/mL and for EP2 in the range of 0.125–8 ng/mL. The accuracy estimates ranged between 98% and 108% for EP1 and between 90% and 110% for EP2 in the reproducible range mentioned above. Intermediate precision (within-plate R.S.D.) in the same range was within 26% and 17% for the EP1 and EP2 bioassays, respectively. The validated bioassays for EP1 and EP2 were utilized to quantitatively analyze serum samples from a pharmacokinetic study conducted to compare the profiles of the two compounds in Sprague–Dawley rats.

Keywords: 32D cell line; Erythropoietin; Proliferation; Serum-based bioassay; Quantitative cell-based bioassay

1. Introduction

In vitro cell-based bioassays conducted in biological matrices provide a useful mechanism for the quantification of active drug levels in samples derived from pharmacokinetic (PK) or toxicokinetic (TK) studies. While immunoassays may measure biologically active drug, inactive drug and metabolites generated *in vivo* [1,2], cell-based bioassays provide an estimate of biologically active drug levels [3,4,8]. For quantitative purposes, a typical bioassay utilizes a cell line that responds to the drug with a measurable response (OD, counts, etc.) [4–8]. Quantitative uses of *in vitro* bioassays include potency evaluations of manufactured drug lots [8,9] or the measurement of active drug concentration in samples derived from PK or TK studies. For potency measurements, the bioassays are conducted in cell culture medium, an environment that has been optimized for cell growth. However, for the analysis of biological samples, a bioassay has to be adapted to function in the presence of test animal serum. This adaptation poses a challenge to the assay developer since cell lines can be extremely sensitive to changes in their routine culture medium. The introduction of serum or plasma from a species other than the one that the cell line is derived from

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into the culture medium must be done judiciously to maintain responsiveness to the drug.

Drug-induced change in the proliferation of a cell line is the most convenient assay readout if the drug product is a growth factor or a cytokine. Changes in cell proliferation can be easily measured using either tritiated thymidine [10–12] or by choosing from a variety of non-radioactive dyes, i.e. methylthiotetrazole or MTS [13] (Promega, Madison WI), Alamar Blue [14] (Biosource International, Camarillo, CA), etc., or by using reagents that measure ATP levels of live cells, i.e. CellTiter-Glo (Promega) or ViaLight (Cambrex Corp., East Rutherford, NJ) [15]. Other drug-induced responses that could be selected as bioassay readout include the induction of a protein that could be measured by an ELISA. Alternatively, the induced protein may also be measured at the mRNA level [6,7] using currently available technology, i.e. Quantigene (Bayer Corp., Tarrytown, NY).

The bioassay described in this paper utilized EP1- and EP2-induced proliferation of the 32D cell line, measured by tritiated thymidine incorporation into the cellular DNA as readout. Proliferation was considered to be a suitable surrogate endpoint *in vitro* for EP1 and EP2 bioactivity measurement since it closely resembled their desired function *in vivo*. The bioassay was adapted to Sprague–Dawley rat serum to facilitate the analysis of samples from a PK study conducted with EP1 and EP2. The method was subsequently validated for its robustness, linearity, accuracy, precision, sensitivity and specificity in accordance with the ICH and FDA guidelines [17–19] using good laboratory practices (GLPs) [20].

2. Experimental

2.1. EP1 and EP2

Both EP1 and EP2 are recombinant proteins with EP1 being the parent compound. EP2 was derived by amino acid substitution of EP1 with subsequent addition of carbohydrate sidechains in order to increase the residence time of the parent compound. The alteration in the protein structure reduced the *in vitro* biological activity of EP2 on a molar basis and rendered it undetectable by the ELISA used for the detection of EP1. Hence, cell-based assays useful for measuring the biological activity of EP1 and EP2 were validated for measuring the concentrations of EP1 and EP2 from a PK study comparing the half-lives of the two compounds *in vivo*.

2.2. Materials

RPMI 1640 medium, 100× penicillin/streptomycin/L-glutamine and Trypan Blue were purchased from Gibco-BRL (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). [Methyl-^{3}H]thymidine was from Amersham Biosciences Corp (Piscataway, NJ). Murine interlukin-3 (mIL-3) and biotinylated anti-human EPO receptor antibody were obtained from R&D Systems (Minneapolis, MN). Pooled rat serum was purchased from Bioreclamation Inc. (Hicksville, NY). Enzymatic Amplification Staining (EAS) kit was obtained from FlowAmp (Cleveland, OH). This staining kit contains streptavidin–HRP, EAS amplifier, EAS amplifier medium and streptavidin–FITC. Recombinant human EPO (rHuEPO), EP1, EP2 and rabbit anti-EP1 neutralizing antibody were provided by Amgen Inc. (Thousand Oaks, CA).

2.3. Maintenance of cell cultures

The sub clone of murine 32D cell line expressing the transfected human EPO receptor was obtained from the Amgen Cell Bank (referred to as 32D cells hereafter). Cells were maintained in vented cap, canted neck, cell culture flasks at 37 °C, 5% CO_2, and 95% r.h. The growth medium consisted of RPMI 1640 supplemented with 15% heat-inactivated FBS, 2 mM L-glutamine and 1% (v/v) penicillin/streptomycin mixture. Because the cells were dependent on rHuEPO for growth, the growth medium was supplemented with 10 U/mL of rHuEPO. Cells were subcultured two to three times a week to maintain cell densities between 5×10^4 and 1×10^6 cells/mL. Healthy growing cells were generally 98–100% viable as measured by Trypan Blue exclusion method. Cells were banked at passage 9 and used in assays for up to 40 days after thawing, at which point the old cells were discarded and a vial of frozen cells was thawed and expanded in culture.

2.4. Labeling of EPO receptor for FACS analysis

32D cells were harvested by centrifugation at 200–300 × *g*, washed once with PBS containing 2% FBS (referred to as staining buffer) and spun down by centrifugation. Subsequently, the cells were resuspended in staining buffer and the viable cell count was determined using Trypan Blue. One million cells contained in 50 μL staining buffer were incubated with 0.5 μg biotinylated anti-human EPO receptor antibody in a polystyrene tube at room temperature for 10 min. The cells were washed twice with staining buffer (2 mL/tube) and spun down by centrifugation. One microliter of streptavidin–HRP diluted in 50 μL staining buffer was added to the cell pellet and incubated at room temperature for 10 min. Subsequently, the cells were washed again: four times with staining buffer and once with PBS. Cells were incubated with 2.5 μL EAS amplifier diluted in 50 μL EAS amplifier medium at room temperature for 10 min. After washing twice with staining buffer, the cells were incubated with 50 μL streptavidin–FITC (1:100 diluted in staining buffer) at RT for 10 min in the dark. The unbound streptavidin–FITC was removed by washing the cells twice with staining buffer. The cells were finally resuspended in 500 μL staining buffer and analyzed using the FACS Calibur (BD Biosciences, San Jose, CA).

2.5. Cell proliferation assay

Overnight-grown 32D cells were harvested and washed twice with growth medium lacking rHuEPO in order to arrest cell growth. The cells were washed by transferring the contents of a flask into a polypropylene tube and centrifuged at 200–300 × *g*. The supernatant was discarded and the cell pellet

was resuspended in growth medium and recentrifuged. The final cell pellet was resuspended, the cell density was adjusted to 5×10^5 cells/mL in the absence of EPO. Cells were staged by incubating at 37 °C, 5% CO_2 and 95% r.h. for 16–24 h. Subsequently, the cells were centrifuged, resuspended in fresh growth medium and counted. Twenty thousand staged 32D cells in 100 μL volume were incubated with 100 μL prepared testing sample in a 96-well culture plate for 44 ± 1 h at 37 °C, 5% CO_2 and 95% r.h. Each sample preparation was tested in triplicate. At the end of the incubation, 2 μCi [methyl-^{3}H]thymidine diluted in 50 μL growth medium were added to each experimental well and the plate was incubated for an additional 4 ± 1 h in the incubator. The contents of the plate were harvested onto a filter mat using a plate harvester (Packard Instrument, Downers Grove, IL). The filter mat was air dried and loaded on a beta ray counter (9600-Matrix Counter, Packard) and counted for 6 min.

2.6. Pharmacokinetic study design and sample analysis

Male Sprague–Dawley rats ($n = 10$) were randomly assigned to four groups of two or three rats per group. Animals in groups 1 and 2 ($n = 2$/group) received single intravenous (IV) or subcutaneous (SC) bolus injections of EP1 at 300 μg/kg, respectively. Animals in groups 3 and 4 ($n = 3$/group) received single IV or SC bolus injections of EP2 at 300 μg/kg, respectively. Sampling times for IV and SC EP1-treated animals were predose, 5 (IV only), 15 and 30 min; 1, 2, 4, 8, 12, 24, 48, 72, 96, 144, 192 and 240 h postdose. Sampling times for EP2-treated animals were predose, 5 (IV only), 15 and 30 min; 1, 2, 4, 8, 12, 24, 48, 72, 96, 144, 192, 240, 288 and 336 h postdose. After blood collection into serum separator tubes, samples were allowed to clot on wet ice, and then centrifuged at 2–8 °C. The serum was removed and stored at −70 °C until analysis.

To conduct the bioassay, rat serum samples were thawed at room temperature and diluted at 1:2.5 with growth medium to obtain a 40% serum concentration. Further dilutions, if needed, were made using 40% pooled rat serum as diluent. The pooled rat serum had previously been screened in a bioassay for minimal background values. One hundred microliters of the diluted sample were incubated with 100 μL 2×10^4 staged 32D cells for 44 ± 1 h. This dilution resulted in a final concentration of 20% rat serum, which represented the assay matrix. Radiolabelled thymidine addition, cell harvesting and filter mat counting procedures were performed according to the details outlined previously.

For the analysis of EP1 test samples, each plate carried an EP1 standard curve in the range of 0.039–2.5 ng/mL, and quality control (QC) samples at 0.1, 0.5 and 1 ng/mL in 20% rat serum. QC samples were prepared independently of standards and were used for the purpose of accepting or rejecting the analytical run. All standards and QC samples were prepared in 40% pooled rat serum at a 2× higher concentration than that mentioned above. One hundred microliters of each standard curve and QC solution were mixed with 100 μL 2×10^4 staged 32D cells to initiate the assay. An acceptable assay was one in which six of the eight standards and six of the nine QC replicates predicted within 30% of nominal with a coefficient of variance of less than 25%. For each animal, the predose sample along with its corresponding postdose samples were diluted at the same dilution factor(s), and assayed on the same plate. The mean back-calculated concentration value obtained with any predose sample was subtracted from the results of its corresponding postdose samples. The dilution was determined by a predicted simulation of the PK profile. Samples were diluted to the (predicted) concentration range of 0.039–2.5 ng/mL. For the IV injected animal, samples at different time points were diluted at 1:10, 1:100, 1:1000 or 1:5000. For the SC injected animal, samples were diluted at 1:10, 1:20, 1:100, 1:200 or 1:500. In the event that the initial dilution was inappropriate, the sample was retested at another dilution that fell within 0.039–2.5 ng/mL in the assay.

For the analysis of EP2 test samples, each plate carried an EP2 standard curve in the range of 0.125–32 ng/mL, and QC samples at 0.5, 2 and 4 ng/mL in 20% rat serum. All standards and QC samples were prepared in triplicate. An acceptable assay was one in which six of the seven standards (in the 0.125–8 ng/mL range) and six of the nine QC replicates predicted within 25% of nominal with a coefficient of variance of less than 25%. For each animal, the predose sample along with its corresponding postdose samples were diluted at the same dilution factor(s), and assayed on the same plate. The concentration value obtained with any predose sample was subtracted from the results of its corresponding postdose samples. Sample dilutions were performed based on predicted EP2 PK profiles. Samples were diluted to the concentration range of 0.125–8 ng/mL. For the IV injected animal, samples at different time points were diluted differently at 1:10, 1:100, 1:1000 and 1:5000. For the SC injected animal, samples were diluted at 1:10, 1:20, 1:100 and 1:1000.

3. Results

3.1. Expression of cell surface EPO receptors

32D cells express low levels of transfected HuEPO receptors that were difficult to detect using the standard biotin/avidin staining method (data not shown). The EAS kit utilizes an enzymatic amplification mechanism to achieve signal enhancement and therefore was successful in detecting the transfected

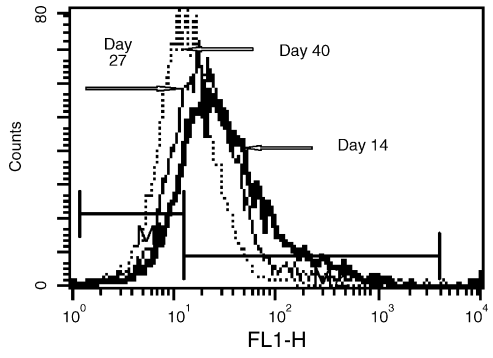

Fig. 1. Effect of cell culture age on EPO receptor expression in 32D cells. Three vials of 32D cells were revived in 13-day intervals. Cells were cultured to 14, 27 and 40 days old and then harvested on the same day. One million 32D cells at each cell age were stained with biotinlyted anti-HuEPO receptor antibody and EAS amplification reagents as described in Section 2.4. The fluorescent signal stained for the transfected HuEPO receptors was analyzed on FL1 channel.

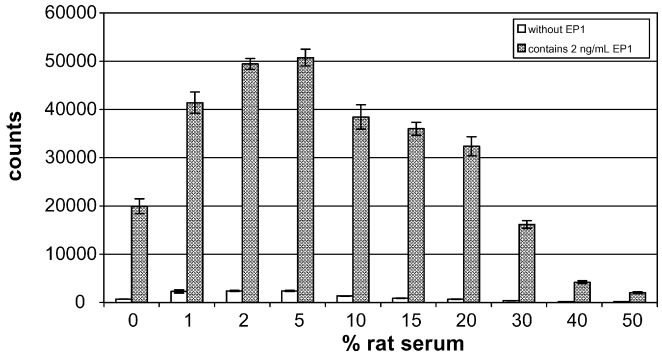

Fig. 2. Tolerance of 32D cells to rat serum. Staged cells (2×10^4/well) were incubated with (i) pooled rat serum (open bar) or (ii) pooled rat serum containing 2 ng/mL EP1 for 48 h in a 37 °C incubator. Proliferation was measured using tritiated thymidine incorporation into cellular DNA. Each condition was tested in triplicate. The error bar represents the standard deviation of each triplicate measurement.

receptors. The histogram for the day-14 cells is wider than that obtained with the day-40 cells and the antibody staining intensity decreased as the cells aged (Fig. 1). These data suggest that the transfected HuEPO receptor expression is more variable in younger cells than older cells. The cells with high expression were gradually lost, and overall receptor expression decreased as the cells aged. The effect of reduced receptor expression was evaluated during assay validation while determining the robustness of the assay.

3.2. Selection of optimum serum matrix

The proliferative response of the 32D cells to EP1 was examined in the presence of 0%, 1%, 2%, 5%, 10%, 15%, 20%, 30%, 40%, and 50% rat serum. Minimal proliferation was observed in the absence of EP1 (Fig. 2). A robust response to EP1 was observed in the presence of 1–20% rat serum. The response to EP1 was markedly increased in the presence of rat serum indicating synergistic activity in rat serum that potentiated the effect of EP1 on the murine 32D cell line. Concentrations of rat serum higher than 20% inhibited the ability of the cells to respond to EP1, a result that could be attributable to serum-mediated cytotoxicity. To maximize assay sensitivity, 20% rat serum was selected as the final assay matrix for the bioassay.

3.3. EP1 and EP2 bioassay validation

Each 96-well microplate assay consisted of eight or nine non-zero standard curve points and three QC samples, each run in triplicate. Multiple plates were tested over 4–5 days. Statistical analyses were performed on the data generated to determine the appropriateness of a four-parameter logistic (4PL) regression function [21] to describe the dose–response curve, determine the linearity and range of reliable response of the dose–response curve, evaluate the accuracy and precision of the standards and QC samples, and estimate the limit of quantitation (LOQ) from the standard curves for the two assays. The LOQ estimate was based on the lowest standard curve concentration in the EP1 and EP2 curves that yielded overall mean accuracy and R.S.D. estimates within 20%. The 4PL regression function was utilized to determine the relationship between counts and concentration of EP1 or EP2. Criteria for acceptance of the selected regression function included: $r^2 \geq 0.950$, significance of the regression terms included in the model ($p < 0.05$), and deviations of mean predicted standard curve concentrations within ±20% to 25% of nominal level.

3.3.1. Linearity and range of reliable response

3.3.1.1. EP1 bioassay validation results. The 4PL function provided a good fit in the range from 0.039 to 2.5 ng/mL (Table 1). The r^2 values for the 12 curves were ≥0.989, and no significant departure from the selected model [lack of fit (LOF) p-value ≥ 0.017] was seen, except for 1 curve (day 4, plate no.

Table 1
Summary of individual EP1 standard curve regression results using the four-parameter logistic function

Assay date	Plate	b	ED_{50} (ng/mL)	Min (counts)	Max (counts)	LOF p-value	r^2
Day 1	1-1	1.288	0.947	497	75606	0.271	0.995
	1-2	1.568	0.676	1303	65845	0.379	0.994
	1-3	1.394	0.706	545	61223	0.067	0.991
Day 2	2-1	1.084	0.769	1663	53965	0.965	0.996
	2-2	1.187	0.679	2252	54859	0.629	0.989
	2-3	0.929	0.883	0	63262	0.024	0.993
Day 3	3-1	1.031	0.97	1341	59932	0.112	0.995
	3-2	1.434	0.584	2810	50594	0.263	0.998
	3-3	1.202	0.687	1442	55260	0.819	0.996
Day 4	4-1	1.082	0.784	1424	57494	0.00	0.995
	4-2	1.224	0.671	2051	50905	0.861	0.995
	4-3	1.212	0.673	1682	52618	0.912	0.991

EP1 standard curves (of final concentration 0.039–2.5 ng/mL) were prepared fresh on the day of each experiment. The standard curves were prepared by adding the appropriate amount of EP1 into 40% pooled rat serum. One hundred microliters of each standard curve solution were added into the wells of a 96-well microplate followed by addition of 100 μL of cell suspension at 2×10^5/mL. The mixture was incubated for 48 h in a 37 °C incubator and proliferation was measured as described in Section 2.5. The four-parameter logistic regression function is described as counts = max + {(min − max)/[1 + (conc/ED_{50})^b]}, where 'max' is as estimate of the maximum counts, 'conc' is the EP1 concentration (0.039–2.5 ng/mL), 'ED_{50}' is the estimate of the concentration corresponding to the midpoint of the function and b is proportional to the slope.

Table 2
Summary of accuracy and precision assessments for EP1 standard curves

	Concentration (ng/mL) ($n = 36$)							
	0.039	0.078	0.156	0.313	0.625	1.25	2.0	2.5
Accuracy								
Mean (ng/mL)	0.04	0.077	0.155	0.313	0.63	1.272	1.98	2.68
%deviation	2.80	−1.40	−0.90	0.30	0.80	1.70	−1.10	7.40
Precision (R.S.D.)								
Between-day	0	0	0	0.42	0	0.81	0	3
Between-plate	10.32	0	3.06	0	0	0	5.16	0
Within-plate	14.01	7.03	6.35	8.64	6.82	15.07	18.68	25.51

1). For each of the 12 curves, the estimate for maximum counts, slope (b) and ED_{50} were all significantly different from zero. Taken together, the significance of the parameters, the high r^2 values, and the goodness of fit test supported use of the 4PL function in the range from 0.39 to 2.5 ng/mL.

Overall accuracy estimates (mean %deviation from nominal) across the 12 curves were within ±8% of nominal value (Table 2). Between-day and between-plate precision estimates were within 11% (R.S.D.). Within-plate precision estimates ranged from 6% at 0.156 ng/mL to 26% at 2.5 ng/mL. Overall accuracy was within ±2% of nominal for the 0.1, 0.5 and 1.0 ng/mL QC samples (Table 3). Between-day, between-plate and within-plate precision estimates were within 13% (R.S.D.) (Table 3). Total variance estimates were within 17% CV. The limit of quantification of the EP1 bioassay was observed to be 0.039 ng/mL, based on the accuracy and precision assessment for the standards (Table 2). The 0.039 ng/mL standard was the first standard with overall mean accuracy and precision estimates within 20%.

3.3.1.2. EP2 bioassay validation results. For all 15 curves, the r^2 values were ≥0.988 (Table 4). The p-value for testing the goodness of fit was greater than 0.017 (0.05/3 for curves generated from assays that contained three plates) or 0.008 (0.05/6 for curves generated from assays that contained six plates) for 10 of the 15 curves. Five curves that had p-values <0.017 or 0.008 indicated departure from model fit. Systemic inspection showed that the departure occurred only at the asymptotes and therefore was of little concern. The estimate of slope (b), ED_{50} and maximum were significantly different from zero for all curves. A representative 4PL fitting for the EP2 standards is demonstrated in Fig. 3 using day-4 data presented in Table 4.

Within the range of 0.125–8 ng/mL, the mean %deviations across curves were within ±10% of nominal values (Table 5). Between-day, between-plate and within-plate precision estimates were within 17% in the same concentration range. The accuracy and precision estimates were more variable in the range of 16–32 ng/mL. Fig. 4 demonstrates the accuracy variability in different concentration ranges of the curve using the five standard curves that showed lack of fit to the 4PL function (Table 4). It shows that the accuracy in the mid range of the curve was well retained within ±25% of the nominal values for all five curves. However, at 16 and 32 ng/mL, four of the five curves had at least one predicted concentration deviated more than 25% from the nominal values. This further validates the observation that the departure of these five curves from 4PL occurred only at the upper asymptote and therefore resulted in no impact to the concentration calculation in the mid range of the curves.

Table 3
Summary of accuracy and precision assessments for EP1 QC samples ($n = 45$)

	0.1 ng/mL	0.5 ng/mL	1 ng/mL
Accuracy			
Mean (ng/mL)	0.098	0.509	1.01
%deviation	−1.80	1.70	0.90
Precision (R.S.D.)			
Between-day	0	0	0
Between-plate	5.1	9.62	12.24
Within-plate	6.5	8.1	11.54
Total	8.06	12.24	16.6

For Tables 2 and 3, accuracy is expressed as %deviation, which is calculated as: (mean calculated value/nominal value) × 100 − 100. Estimates of overall between-day, between-plate and within-plate precision were calculated at each EP1 concentration level for standard curves and QC samples. Variance components methods [16] were utilized for deriving precision estimates. Since the precision estimates were calculated in a hierarchical manner, it was possible that once the within-plate precision was calculated, there was no variation 'left over' to attribute to plate-to-plate or day-to-day variation. In these cases, the between-day or between-plate precision estimates were set to '0'.

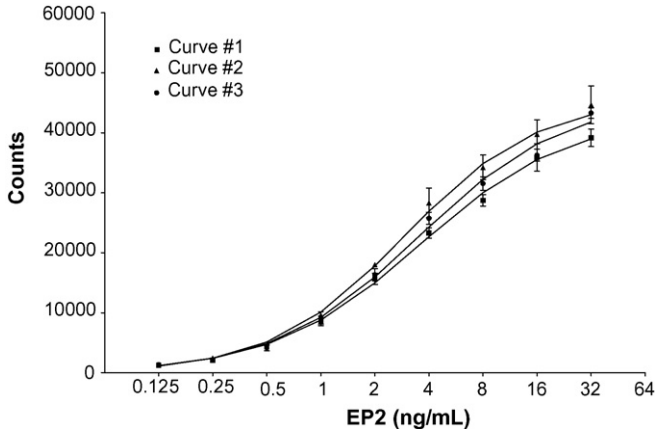

Fig. 3. The 4PL fitting for EP2 standards. Fitting of EP2 standards to the 4PL was performed using SAS software Version 6.12. The graph shows the max, ED_{50} and slope of EP2 standard curves were significantly different from zero, and 4PL appropriately described the relationship between EP2 concentrations and counts.

Table 4
Summary of individual EP2 standard curve regression results using the four-parameter logistic function

Assay date	Plate number	b	ED_{50} (ng/mL)	Min (counts)	Max (counts)	LOF p-value*	r^2
Day 1	1-1	1.469	2.349	3968	67772	0.011*	0.993
	1-2	0.904	4.163	0	107763	0.002*	0.994
	1-3	0.941	3.439	0	80556	0.001*	0.996
Day 2	2-1	0.848	3.876	0	89018	0.037	0.993
	2-2	0.96	2.448	0	84150	0.312	0.995
	2-3	0.984	2.452	173	89047	0.941	0.996
Day 3	3-1	1.13	3.665	2322	91122	0.585	0.994
	3-2	1.149	3.505	3304	93359	0.848	0.996
	3-3	1.067	4.089	2618	96136	0.026	0.994
	3-4	1.117	3.608	2745	95392	0.739	0.993
	3-5	1.008	3.816	1643	105915	0.783	0.996
	3-6	1.027	3.394	1304	95244	0.000*	0.994
Day 4	4-1	1.062	3.579	0	42778	0.222	0.994
	4-2	1.169	2.93	0	45629	0.111	0.988
	4-3	1.082	3.559	0	45676	0.000*	0.993

EP2 standard curves (of final concentration 0.125–32 ng/mL) were prepared fresh on the day of each experiment. The standard curves were prepared by adding the appropriate amount of EP2 into 40% pooled rat serum. One hundred microliters of each standard curve solution were added into the wells of a 96-well microplate followed by addition of 100 μL of cell suspension (2×10^5/mL). The mixture was incubated for 48 h in a 37 °C incubator and proliferation was measured as described in Section 2.5. The 4PL is described as counts = max + {(min − max)/[1 + (conc/ED_{50})^b]}, where 'max' is the estimate of the maximum counts, 'conc' is the EP2 concentration (0.125–32 ng/mL), 'ED_{50}' is the estimate of the dose corresponding to the midpoint of the function and b is proportional to the slope.

* Significant departure from selected model was noted if $p < 0.05/n$, where n is the number of plates run each day.

Table 5
Summary of accuracy and precision assessment for EP2 standard curves

	Concentration (ng/mL) (n = 45)								
	0.125	0.25	0.5	1	2	4	8	16	32
Accuracy									
Mean (ng/mL)	0.14	0.23	0.48	1.01	2.04	4.22	7.49	17.9	38.19
%deviation	9.50	−9.80	−4.80	0.70	2.00	5.50	−6.40	11.80	19.30
Precision (R.S.D.)									
Between-day	2.33	0	3.2	4.08	1.43	2.71	0	8.59	0
Between-plate	16.09	4.46	3.49	0	3.31	0	9.82	0	0
Within-plate	7.88	6.8	9.6	9.55	6.67	11.38	16.47	33.71	69.11

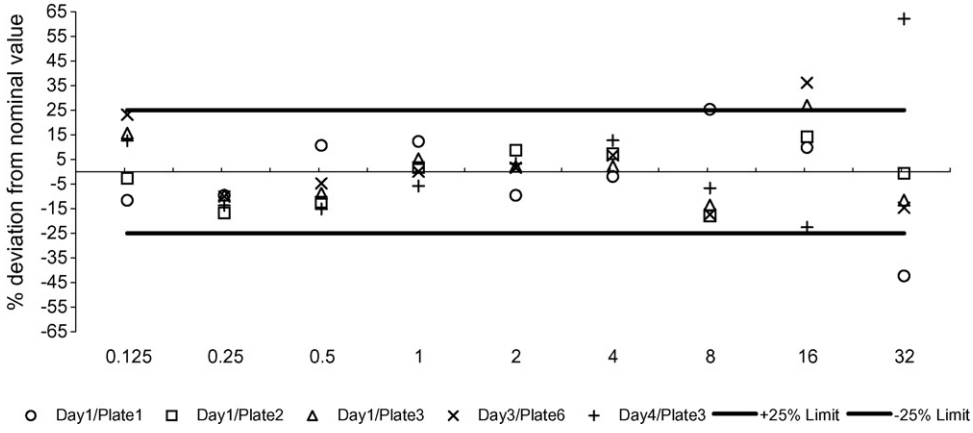

Fig. 4. Accuracy evaluation for EP2 standard curves. The %deviation from nominal value at all concentration levels was plotted for five standard curves that had significant departure from 4PL model ($p < 0.05/n$). The solid lines show positive and negative 25% deviation range from nominal value. X-axis represents EP2 concentration (ng/mL) and is graphed in the middle of the ±25% range with EP2 concentration labels at the bottom. Data indicates that even with curves that showed lack of fit to the 4PL model, only the asymptote level of 16 and 32 ng/mL yielded greater than 25% deviation from nominal values.

Table 6
Summary of accuracy and precision assessment for EP2 QC samples ($n = 45$)

	0.5 ng/mL	2 ng/mL	8 ng/mL[a]
Accuracy			
Mean (ng/mL)	0.53	2.2	7.19
%deviation	5.30	10.20	−10.1
Precision (R.S.D.)			
Between-day	8.92	0.00	17.76
Between-plate	7.32	7.85	20.57
Within-plate	10.29	10.14	17.39

For Tables 5 and 6, accuracy is expressed as %deviation, which is calculated as: (mean calculated value/nominal value) × 100 − 100. Estimates of overall between-day, between-plate and within-plate precision were calculated at each EP2 concentration level for standard curves and QC samples. Variance components methods [16] were utilized for deriving precision estimates. Since the precision estimates were calculated in a hierarchical manner, it was possible that once the within-plate precision was calculated, there was no variation 'left over' to attribute to plate-to-plate or day-to-day variation. In these cases, the between-day or between-plate precision estimates were set to '0'.

[a] $n = 36$.

In summary, the statistical analysis indicated that the 4PL regression model provided an adequate fit to the proliferation data for EP2 in 0.125–8 ng/mL concentration ranges.

The overall accuracy estimates across the three QC concentrations were within 11% of nominal (Table 6). Between-day, between-plate and within-plate precision estimates were within 21%. The limit of quantification of the EP2 bioassay was observed to be 0.125 ng/mL, based on the accuracy and precision assessment for the standards presented in Table 5. The 0.125 ng/mL standard was the first standard with overall mean accuracy and precision estimates within 20%.

3.3.2. Specificity

The specificity of 32D cell proliferation responding to EP1 stimulation was demonstrated by the inhibitory effect of a rabbit anti-EP1 antibody. Increasing concentrations of the anti-EP1 antibody showed inhibition of EP1-induced cell proliferation, and at 50–100 ng/mL, the antibody completely abolished cell proliferation (Fig. 5).

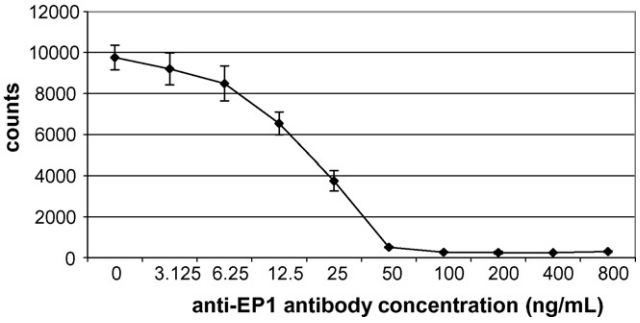

Fig. 5. Inhibitory effect of anti-EP1 antibody on EP1-induced proliferation. The antibody was diluted and incubated with EP1 for at least 30 min at room temperature before incubation with 32D cells. The final concentrations of antibody were 0, 3.125, 6.25, 12.5, 25, 50, 100, 200, 400 and 800 ng/mL. The final concentration of EP1 was 2 ng/mL. Cell proliferation response was measured as described in Section 2.5.

The 32D cell line is known to express receptors for murine interleukin-3 (mIL-3) and thrombopoietin (TPO) and to respond to these cytokines with proliferation. It was important to determine if 20% rat serum possessed basal proliferative activity towards the 32D cells that could interfere with the measurement of EP1 and EP2 levels in test samples. Eighteen blank serum samples were tested and none was observed to yield counts equivalent or higher than those induced by 0.125 ng/mL EP2, the limit of quantification for the assay (Fig. 6). It was concluded that rat serum had a negligible influence on the assay and that the assay was specific for the detection of EPO-like molecules.

3.3.3. Robustness

Cell lines, especially transfected ones, undergo spontaneous mutations in culture resulting in altered responsiveness to the growth factor or cytokine on which they depend. Flow cytometric analysis showed that 32D cells lose transfected huEPO receptors as they age (Fig. 1). To determine whether the observed changes at the receptor level interfered with the ability of the cells to respond to EP1 or EP2, EP2 standard curves and QC samples were prepared and tested with 32D cell cultures at day 14, day 27 and day 40. The regression results showed that the 4PL function provided an adequate fit to the standard curves for

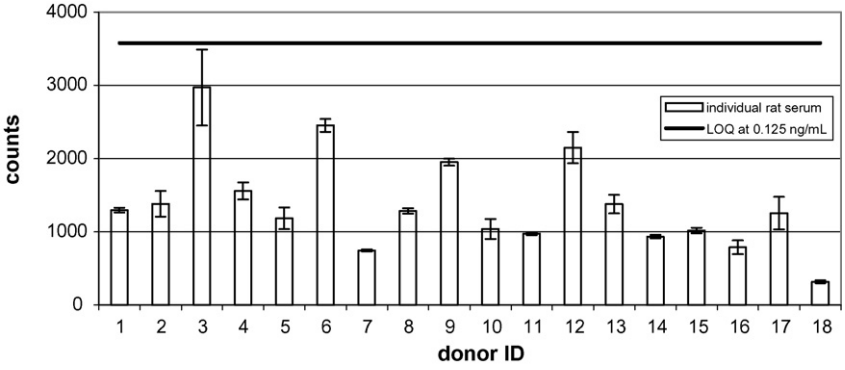

Fig. 6. Effect of individual rat serum on 32D cell proliferation response. Eighteen adult Sprague–Dawley rat sera were individually diluted with growth medium and incubated with 32D cells at final concentration of 20% (v/v). Cell proliferation assay was conducted on three plates as described in Section 2.5. Each plate also included an EP2 standard curve and three QC samples at 0.5, 2, and 4 ng/mL prepared in pooled rat serum to pass or fail the assay. Error bar represent standard deviation of triplicate measurement.

Table 7
Regression results of EP2 standard curves obtained using different age of cells

Cell age	Plate number	b	ED_{50} (ng/mL)	Min (counts)	Max (counts)	LOF p-value	r^2
Day 14	1-1	1.231	6.747	0	57027	0.539	0.991
	1-2	1.261	5.992	0	64945	0.07	0.994
	1-3	1.342	5.315	0	66801	0.836	0.994
Day 27	2-1	0.989	6.418	0	91610	0.234	0.996
	2-2	1.046	6.069	0	88139	0.131	0.999
	2-3	1.142	4.588	0	80917	0.133	0.998
Day 40	3-1	1.004	3.511	0	116087	0.296	0.995
	3-2	1.033	3.663	319	105731	0.792	0.996
	3-3	1.117	3.272	880	101592	0.072	0.997

EP2 standard curves were prepared fresh at the day of the experiment. Final concentrations of EP2 standard curve were 0.125, 0.25, 0.5, 1, 2, 4, 8, 16 and 32 ng/mL. One hundred microliters of each standard curve solution were added into the wells of 96-well microplates followed by addition of 100 μL of cell suspension of 2×10^5/mL. Cells were at culture age 14, 27 and 40 days old. For each cell age, three plates were set-up with one standard curve plated on each plate. Cells and the EP2 standard curve were incubated for 48 h in a 37°C incubator and proliferation was measured as described in Section 2.5. The 4PL is utilized to describe the relationship between the cell proliferation response (counts) and EP2 concentrations. The regression results were utilized to determine if the cell age affected the shapes of the curves.

cells of all ages (Table 7). The r^2 for all curves were ≥0.991 and no systemic departure from the 4PL fit was observed with any of the curves ($p > 0.070$). The mean %deviations were within 22% of nominal values at 0.125–8 ng/mL for all cell ages evaluated, and the precision estimates were within 16% in the same concentration range for all cells (Table 8). Cells at all ages tested demonstrated a dose-dependent response to EP2. However, it was noticed that curves generated with cells at day 40 yielded lower ED_{50} values than those observed with curves prepared with cells at days 14 and 27. Since flow cytometric analysis of these cell preparations shown in Fig. 1 indicated that these cells show reduced EPOR expression with age, the lower ED_{50} values obtained with day 40 cells indicated that these cells might be undergoing a genetic drift that renders their proliferation EPO-independent (Fig. 7).

Overall estimates for accuracy ranged from 0.8% to 24% across QC levels at all ages (Table 9). Only one QC sample of 4 ng/mL prepared with cells at day 14 deviated significantly at 34% above nominal; however, the 95% confidence interval for the mean (5.05–5.63 ng/mL) was within the nominal range of 3.8–6.2 ng/mL. Estimates of between-plate and within-plate precision were within 16% across QC levels at all cell ages.

Table 8
Summary of accuracy and precision for the EP2 standards at different cell ages

	Concentration (ng/mL)						
	0.125	0.25	0.5	1	2	4	8
Day 14 ($n = 9$)							
Accuracy							
Mean	0.13	0.22	0.41	0.88	1.94	4.37	7.86
%deviation	3.30	−14.00	−17.60	−11.70	−3.20	9.40	−1.80
Precision (R.S.D.)							
Between-plate	0.15	0	6.12	0	0	0	0
Within-plate	15.78	15.28	8.48	7.96	11.1	9.92	12
Day 27 ($n = 9$)							
Accuracy							
Mean	0.13	0.21	0.45	0.95	2.07	4.19	7.85
%deviation	4.30	−14.60	−10.10	−5.30	3.70	4.70	−1.90
Precision (R.S.D.)							
Between-plate	10.63	14.31	2.24	0	0	0	0
Within-plate	5.2	12.96	8.11	8.98	8.6	6.02	8.96
Day 40 ($n = 9$)							
Accuracy							
Mean	0.15	0.23	0.48	0.96	2.08	4.19	7.6
%deviation	21.70	−9.8	−4.60	−4.40	4.10	4.80	−5.00
Precision (R.S.D.)							
Between-plate	7.87	0	3.87	0	2.1	0	0
Within-plate	6.9	11.69	7.29	4.72	5.38	7.55	13.68

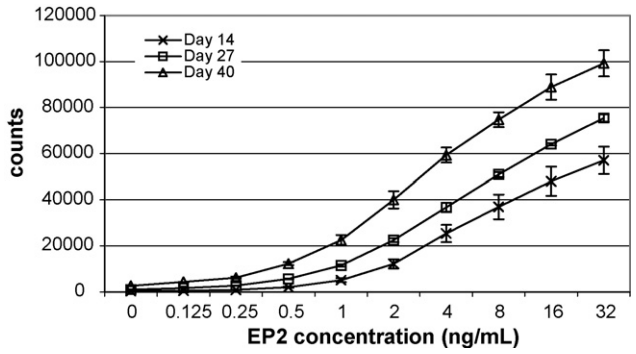

Fig. 7. Cell age effect on EP2-induced proliferation. 32D cells at culture age 14, 27 and 40 days old were incubated with EP2 standard curve at final concentrations of 0.125, 0.25, 0.5, 1, 2, 4, 8, 16 and 32 ng/mL. Cell proliferation was measured as described in Section 2.5. For each cell age, three plates were setup with one standard curve (in triplicate) plated on each plate. The mean counts of the three standard curves at each EP2 concentration level were graphed for each cell age. Error bar represents the standard deviation of each three curves.

The statistical analysis indicated cell age did not significantly affect the accuracy and precision of EP2 QC samples. Since EP1 and EP2 utilize the same signaling pathway to achieve the assay endpoint (proliferation), based on these analyses, 32D cells were used up to 40 days in EP1 and EP2 quantitative assays.

3.4. Comparison of EP1 and EP2 pharmacokinetic profiles

After IV administration, the area under the serum–concentration time curves ($AUC_{(0-\infty)}$) for EP2 increased approximately 1.6-fold compared with EP1. $AUC_{(0-\infty)}$ (mean ± S.D.) were 61,800 ± 16,100 ($n = 3$) and 39,700 ($n = 1$) ng h/mL for EP2 and EP1, respectively, resulting in mean ± S.D. clearance values of 5.07 ± 1.23 and 7.57 mL/h/kg, for EP2 and EP1, respectively. Initial volumes of distribution (V_0) (mean ± S.D.) were 34.9 ± 12.9 and 29.9 mL/kg, respectively, which were similar to plasma volume, and the terminal half-lives were 31.0 ± 7.17 and 17.1 h for EP2 and EP1, respectively. These data indicate that EP2 has an approximate 2-fold longer half-life and 1.5-fold slower clearance than EP1 following IV dosing in rats at a dose of 300 μg/kg (Fig. 8).

After SC administration of EP2 to 3 rats, only 1 profile (animal #9) was considered to be representative of the pharmacokinetics for EP2, thus $n = 1$ for both EP2 and EP1 reported parameters. The EP2 and EP1 profiles peaked at approximately

Table 9
Summary of accuracy and precision assessment for all EP2 QC samples for cells at 14, 27 and 40 days of age

	Concentration (ng/mL) [±25% of nominal range]		
	0.5 [0.38–0.62]	2 [1.5–2.5]	4 [3.8–6.2]
Day 14 ($n = 27$)			
Accuracy			
Mean	0.52	2.46	5.34
%deviation	4.10	23.20	33.50
95% CL	0.51–0.53	2.39–2.54[a]	5.05–5.63
Precision (R.S.D.)			
Between-plate	0	5.53	7.5
Within-plate	6.83	6.48	12.09
Day 27 ($n = 27$)			
Accuracy			
Mean	0.51	2.4	4.95
%deviation	2.50	19.90	23.70
95% CL[b]	0.49–0.54	2.31–2.49	4.74–5.15
Precision (R.S.D.)			
Between-plate	9.53	9.65	5.05
Within-plate	9.09	4.99	9.43
Day 40 ($n = 27$)			
Accuracy			
Mean	0.5	2.25	4.7
%deviation	0.80	12.50	17.40
95% CL	0.48–0.53	2.15–2.35	4.38–5.01
Precision (R.S.D.)			
Between-plate	10.96	9.82	7.42
Within-plate	5.75	8.37	15.82

For Tables 8 and 9, accuracy is expressed as %deviation, which is calculated as: (mean calculated value/nominal value) × 100 − 100. Estimates of between-plate and within-plate precision were calculated at each EP2 concentration level for standard curves and QC samples for each cell ages. Variance components methods [16] were utilized for deriving precision estimates. Since the precision estimates were calculated in a hierarchical manner, it was possible that once the within-plate precision was calculated, there was no variation 'left over' to attribute to plate-to-plate variation. In these cases, the between-plate precision estimates were set to '0'.

[a] Confidence interval outside ± 25% of nominal range.

[b] Confidence limit.

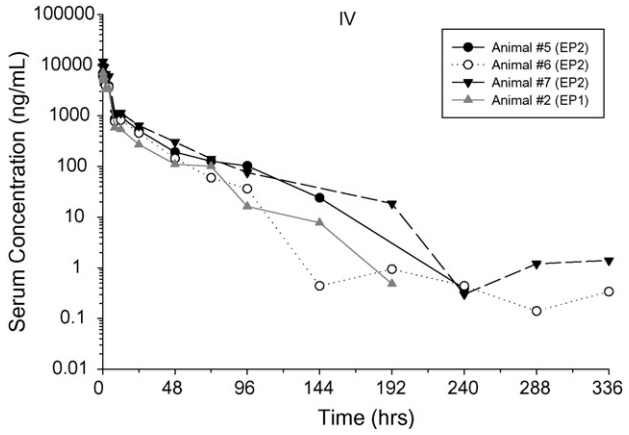

Fig. 8. Individual serum concentration–time profiles after IV administration of 300 μg/kg EP1 or EP2. Serum samples from three EP2 injected animals and one EP1 injected animal were collected at the timepoints indicated on the *X*-axis. Samples at different timepoints were diluted at different dilution factor(s) and tested with its predose at the same dilution on the same plate. Sample concentration was extroplated from the standard curve run on the same plate and subtracted by its corresponding predose concentration. Serum concentration was calculated using sample concentration times dilution factor(s).

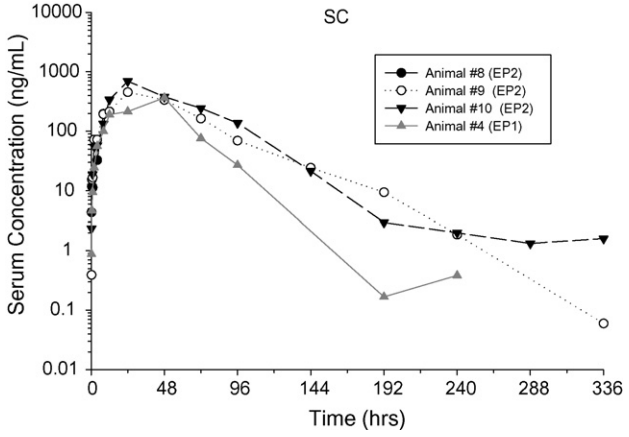

Fig. 9. Individual serum concentration–time profiles after SC administration of 300 μg/kg EP1 or EP2. Sample dilution schema and serum concentration calculation were the same as described in the legend for Fig. 8. Animal #8 died during blood collection at 8 h timepoint. Animals #9 and #10 showed different PK profiles, as #10 had an unexpected slower phase at low EP2 concentrations than #9. Therefore the profile obtained from animal #9 represented a conservative estimate of EP2 profile and was chosen to compare with EP1.

24 and 48 h, respectively, with peak concentrations of 454 and 367 ng/mL, respectively. The terminal half-lives after SC dosing were 28.2 and 13.2 h. For $AUC_{(0-\infty)}$, the ratio (EP2 to EP1) was 1.6. Bioavailability, as estimated from the ratio of $AUC_{(0-\infty),SC}/AUC_{(0-\infty),IV}$ (mean values) were 43% and 42% for EP2 and EP1, respectively. These data indicate that EP2 has an approximate 2-fold longer half-life and 1.6-fold slower clearance than EP1 after SC dosing in rats at a dose of 300 μg/kg (Fig. 9).

4. Discussion

This paper describes the use of an *in vitro* bioassay in a quantitative fashion for the evaluation of two potential therapeutic candidates while under preclinical development. Currently, drug development is focused on bringing therapeutics into the clinic that require less frequent dosing, which saves direct and indirect cost to the medical care system. To achieve this goal, generation of sustained delivery candidates require chemical or other types of modification of the parent molecule that may alter its ability to be measured in an ELISA format used routinely for the parent molecule. In this case, EP2 (the derived product of EP1) could not be measured in the ELISA routinely used for EP1. The PK properties of EP2 as compared to EP1 using the bioassay assisted in allowing preclinical development of EP2 to proceed and justifying the need to develop ELISA reagents for confirming PK studies.

In addition to being labor intensive and tedious, bioassays may be variable compared with other bioanalytic techniques; however, the variability can be minimized by characterization and proper care and maintenance of the cell line. Regular microscopic monitoring of the cell morphology allows early detection of microbial contamination or any unusual changes in cell appearance that could cause assay problems later. Cell subculturing and plating conditions play a vital role in determining how the cells respond to the test article. These conditions must follow a strict protocol to maintain consistency of the cellular response to the test article. If the cells are dependent on a particular growth factor or cytokine for continuous growth, the stability of the agent in the growth medium should be ascertained.

For the bioassay described in this paper it was important to control several conditions to maintain a consistent response. During routine cell culturing, 32D cells were maintained in the density range of 3×10^4 to 1×10^6 cells/mL. Cells were subcultured two or three times per week with fresh growth medium supplemented with 10 U/mL rHuEPO because overgrown 32D cells quickly lose transfected HuEPO receptors. It was also noted that the quality of the pooled rat serum had a critical role in the performance of this particular assay. Pooled rat serum obtained from rats that were bled repeatedly yielded a higher background value in the assay. The possibility of inducing anemia that could result in heightened concentrations of EPO is increased in animals that are frequently bled. Efforts were made to purchase pooled rat serum from a commercial vendor that was willing to provide serum from singly bled rats.

To have a reliable quantitative serum-based bioassay, the assay typically passes through three sequential stages: assay development, prevalidation, and method validation. During assay development, a cell line that exhibits a robust response to the test article is selected. It is recommended that the assay developer choose a stably transfected or a well-characterized cell line. The 32D cell line used in this assay was well characterized as a parent cell line and the work described in this paper evaluating the amount of HuEPO receptor expression at different stages of cell culture provided further information about the changes in the cell line over 40 days. Aging of the cell line did not significantly affect the receptor expression levels or the response of the cell line to EPO, EP1, or EP2 suggesting that the cell line was a stable reagent for the tested time span, thereby providing reliable assay data.

The data generated during the prevalidation stage has not been included in this paper but provided useful information about the capabilities of the assay. Several EP1 and EP2 curves were generated in the selected 20% rat serum matrix and preliminary evaluation of the assay features was made, including minimum and maximum responses of the cells to EP1 and EP2, linear ranges of the curves and the number of standard curve points within the linear range, the r^2- and p-values of the curves, accuracy and precision of the standard curve and QC replicates, and an acceptance criteria that the assay would be able to meet in the routine sample testing scenario.

While no formal regulatory guidelines are available for the validation of serum-based bioassays, the general ICH guidelines [17,18] and FDA guidance for bioanalytical method validation [19] were used for the validation of the EP1 and EP2 bioassays. The validation results suggested that both the EP1 and EP2 bioassays are reliable for the measurement of biologically active drug concentrations in study samples.

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