MATERIALS AND METHODS

Materials

1. Drugs

Brand name	Manufacturer	Lot No.
Epoetin Alpha (Pre-filled syringe) Eprex® 4,000 IU/0.4 ml	Vetter Pharma GmbH., Ravensburg, Germany. Distributor: CILAG AG International. Zug, Switzerland Importer: Janssen-Cilag Ltd., Bangkok, Thailand.	BN 05FS01T Mfd 06/2005 Exp 06/2007
Epoetin Alpha (Pre-filled syringe) Epokine® 4,000 IU	Cheil Jedang Corporation. Seoul, Korea Distributor and Importer: RX Company Ltd., Bangkok, Thailand.	5514 Mfd 25 APR 2005 Exp 24 APR 2007
Epoetin Beta (Pre-filled syringe) Recormon® 2,000 IU (2 doses)	Roche Diagnostics GmbH., Mannheim, Germany. Distributor: F. Hoffman-La Roche Ltd., Basel, Switzerland. Importer: Roche Thailand Ltd., Bangkok, Thailand.	MH6754004 MH6753901 Mfd 04/2004 Exp 04/2006

2. Reagents and chemical substances

- Quantikine® IVD® Erythropoietin ELISA kit (R&D System Inc., Minneapolis, MN, USA).

- Erythropoietin microplate – 96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against rHuEPO.

- Erythropoietin conjugate – a rabbit polyclonal antibody against rHuEPO, conjugated to horseradish peroxidase

- Erythropoietin 0.0 mIU/mL standard – a buffer protein base
- Erythropoietin 2.5 mIU/mL standard – rHuEPO in buffer protein base
- Erythropoietin 5.0 mIU/mL standard – rHuEPO in buffer protein base
- Erythropoietin 20.0 mIU/mL standard – rHuEPO in buffer protein base
- Erythropoietin 50.0 mIU/mL standard – rHuEPO in buffer protein base
- Erythropoietin 100.0 mIU/mL standard – rHuEPO in buffer protein base
- Erythropoietin 200.0 mIU/mL standard – rHuEPO in buffer protein base
- Erythropoietin human serum control 1 (11.70 mIU/mL range: 8.8-12.5)
- Erythropoietin human serum control 2 (40.00 mIU/mL range: 34.0-46.0)
- Erythropoietin human serum control 3 (119.50 mIU/mL range: 102.0-137.0)
- Erythropoietin assay diluent – a buffer protein base
- Specimen diluent – a protein stabilized buffer
- Erythropoietin wash buffer concentrate – a 25-fold concentrate
- Color reagent A – 0.01 N buffered hydrogen peroxide
- Color reagent B – 0.35 g/L tetramethylbenzidine
- Stop solution – 2 N sulfuric acid
- Deionized water

3. Instruments

Multi-channel pipette	Transferpette®, Germany
Micropipette	Gilson, USA
Microplate reader	Tecan Sunrise, Austria
Microplate shaker	PSU 2T, Biosan, Latvia
Microcentrifuge tube	Hycon E1004, USA
Centrifuge	Hermle, Germany
Vortex mixer	Vortex-2 Genie Scientific Inc., USA

Methods

1. Subjects

A total of 12 healthy Thai male volunteers were enrolled and volunteers were screened 14 days before the study day.

Inclusion criteria

1. Healthy nonsmoking male volunteers on the basis of medical history and physical examination, aged between 18-30 years old and body mass index within 18-25 kg/m^2.
2. Complete blood count (CBC) with differential, platelet count, blood glucose, blood urea nitrogen (BUN), creatinine (Cr) and liver function test must be within normal limits.
3. Subject must have negative screening test for HBs-Ag, anti-HBC and anti-HIV (counseling for anti-HIV test will be provided before blood drawing).
4. Subject must be able to give written informed consent and to comply with the protocol requirements.

Exclusion criteria

1. Subject with hypersensitivity to human albumin products and/or mammalian cell-derived products.
2. Subject with history of cardiovascular diseases especially hypertension, pulmonary disease, gastrointestinal disease, renal or hepatic insufficiency, allergic disease, neurologic or endocrine disorders.
3. Subject with known history of recent cigarette smoking, alcohol consumption or drug abuse.
4. Subject taking androgen therapy or taking other drug (except paracetamol) within 2 months before enrollment to the study.
5. Subject previously enrolled in the other drug study within 1 month.

Withdrawal criteria

1. Subject who experiences adverse drug reactions during the study.
2. Subject who cannot comply with the study protocol or voluntarily withdraws from the study.
3. Subject who requires other medication (which interferes with the levels of erythropoietin) during the study period.

2. Ethics

The protocol was approved by the Human Research Ethic Committee of the Faculty of Medicine, Chiang Mai University.

3. Study design

A total of 12 volunteers were recruited and randomly assigned to a single dose, three-treatment, six sequences cross-over study as follows:

Table 1 The randomized schedule of drug administration.

Subject No.	Study visit 1	Study visit 2	Study visit 3
1	Eprex®	Epokine®	Recormon®
2	Eprex®	Recormon®	Epokine®
3	Epokine®	Eprex®	Recormon®
4	Epokine®	Recormon®	Eprex®
5	Recormon®	Eprex®	Epokine®
6	Recormon®	Epokine®	Eprex®
7	Eprex®	Epokine®	Recormon®
8	Eprex®	Recormon®	Epokine®
9	Epokine®	Eprex®	Recormon®
10	Epokine®	Recormon®	Eprex®
11	Recormon®	Eprex®	Epokine®
12	Recormon®	Epokine®	Eprex®

4. Drug administration

On the study day, volunteers were admitted to the Clinical Pharmacology Unit of the Faculty of Medicine, Chiang Mai University at 8:00 AM and the vital signs were recorded. The intravenous catheter was placed on the forearm for the collection of blood samples. After completion of routine physical examination, each volunteer received subcutaneous administration of single dose (4,000 IU) of either Eprex® or Epokine® in the upper arm, Recormon®, on the other hand was administered subcutaneously in equally splitted dose into both upper arms at 9:00 AM by the same physician. Volunteers were instructed to avoid strenuous exertion throughout the study period. The wash out period between each study visit was 21 days. An identical meal and fluid were served during the three study visits. Volunteers were required to refrain from taking other medication, drinking caffeine containing beverages, and alcohol in order to standardize experimental conditions.

5. Blood sample collection

An intravenous catheter connected to an injection plug was used for serial blood sample collection. Venous blood samples (3 mL each) were collected into EDTA coated tubes before each dose and at 1, 2, 4, 8, 12, 15, 18, 24, 48, 72 and 96 h after dose administration. The blood samples were centrifuged at 3,000 rpm for 10 min. Plasma was separated promptly and frozen immediately at –20 °C until assay.

6. Clinical monitoring

Blood pressure and heart rate were monitored before drug administration and at specific time point of blood sample collection. Volunteers were asked to report symptoms of adverse reactions including allergic reactions such as rash, swelling of face or itching.

7. Principle of EPO ELISA

The Quantikine® IVD® Erythropoietin ELISA is based on the double-antibody sandwich method. Microplate wells, precoated with monoclonal (mouse) antibody specific for EPO are incubated with specimen or standard to bind EPO with the

immobilized antibody on the plate. After removing excess specimen or standard, wells are incubated with an anti-EPO polyclonal (rabbit) antibody conjugated to horseradish peroxidase. During the second incubation, the antibody-enzyme conjugate binds to the immobilized EPO. Excess conjugate is removed by washing. A chromogen is added into the wells and is oxidized by the enzyme reaction to form a blue color complex. The reaction is stopped by the addition of 2 N sulfuric acid, which turns the blue color to yellow, which is measured at 450/620 nm. The intensity of color generated is directly proportional to the amount of conjugate bound to the EPO antibody complex. The absorbance of this complex is measured and a standard curve is generated by plotting absorbance versus the concentration of EPO standards. The EPO concentration of unknown specimen is determined by comparing the optical density of the specimen to the standard curve (65).

8. Quantification

Quantification of EPO was determined by using Quantikine® IVD® Erythropoietin ELISA (R&D System Inc. MN, USA) standard curve. The standard curve concentration range was 2.5-200.0 mIU/mL with the correlation coefficients (r) was 0.95 or better (Figure 2) and the lower limit of quantification was 0.6 mIU/mL. The assay was validated and demonstrated of accuracy and precision. Endogenous EPO cross-reacted in this ELISA. Quality control (QC) of plasma samples were control 1 (11.70, range: 8.80-12.50 mIU/mL), control 2 (40.00, range: 34.00-46.00 mIU/mL) and control 3 (119.50, range: 102.00-137.00 mIU/mL).

Standard calibration curves were constructed by plotting the log of absorbance for each standard on the y axis against the log of concentration on the x axis and the best fit line was determined by linear regression. The unknown concentrations of EPO in plasma samples were determined from this calibration curve by inverse prediction.

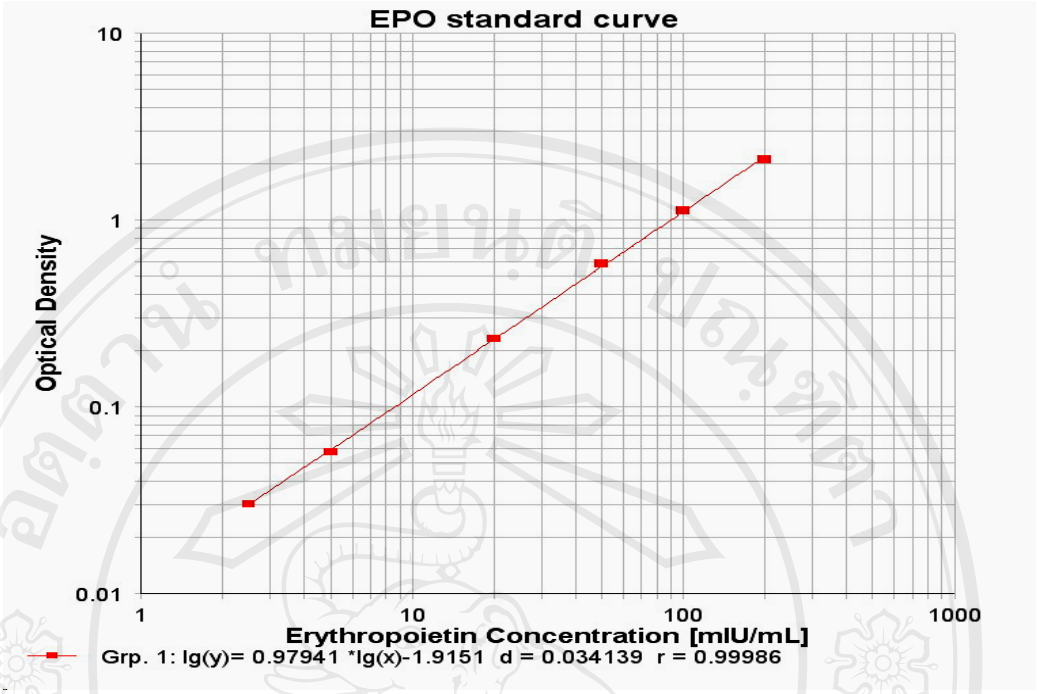

Figure 2 Calibration curve of EPO in human plasma.

9. Precision and accuracy

The inter-day assays were obtained by 8 repeating analysis of three levels of plasma EPO concentration in quality control (QC) samples [control 1 (11.70, range: 8.80-12.50 mIU/mL), control 2 (40.00, range: 34.00-46.00 mIU/mL) and control 3 (119.50, range: 102.00-137.00 mIU/mL)] on 8 different days with concurrent 8 standard calibration curves.

The accuracy of the measured mean value must be within ± 15% of the theoretical value (between 85-115%). The precision represented by coefficient variation value (CV) must be less than 15%.

The accuracy of the measured mean value of QC samples were determined to be 97.70%, 106.26% and 106.28%, respectively (Table 2). The coefficients of variation were determined to be 3.14%, 2.13% and 1.98%, respectively (Table 2).

Table 2 Inter-day assay validation of EPO in plasma.

QC sample	Spiked concentration (mIU/mL)	Measured concentration (mIU/mL)	Accuracy (%)
Control 1 Low	11.70	10.86	92.82
	11.70	11.98	102.39
	11.70	11.68	99.83
	11.70	11.70	100.00
	11.70	11.09	94.79
	11.70	11.49	98.21
	11.70	11.33	96.84
	11.70	11.32	96.75
	Mean	11.43	**97.70**
	S.D.	0.36	
	Precision (CV, %)	**3.14**	
Control 2 Medium	40.00	43.02	107.55
	40.00	43.07	107.68
	40.00	43.40	108.50
	40.00	43.40	108.50
	40.00	42.60	106.50
	40.00	41.12	102.80
	40.00	41.28	103.20
	40.00	42.14	105.35
	Mean	42.50	**106.26**
	S.D.	0.91	
	Precision (CV, %)	**2.13**	
Control 3 High	119.50	132.05	110.50
	119.50	128.49	107.52
	119.50	127.45	106.65
	119.50	127.45	106.65
	119.50	127.17	106.42
	119.50	125.05	104.64
	119.50	125.54	105.05
	119.50	123.78	103.58
	Mean	127.12	**106.38**
	S.D.	2.52	
	Precision (CV, %)	**1.98**	

10.Measurement of EPO concentrations in plasma

Reagent preparation

All reagents were brought to room temperature (20 -25 °C) before use.

- Wash buffer (1X) – Diluted 100 mL of wash buffer concentrate (25X) with deionized water to prepare 2,500 mL of wash buffer (1X)

- Substrate solution – Color reagent A and B should be mixed together in equal volumes within 15 min before use. Protect the final mixture from light and 200 µL of the resultant mixture is required per well.

Sample preparation

Plasma was collected using EDTA as an anticoagulant and centrifuged at 3,000 rpm for 10 min. Plasma was separated promptly and frozen immediately at –20 °C until assay.

Assay procedure

1. Brought all reagents and specimens to room temperature (20-25 °C) before use and gently mixed before pipetting.
2. Pipetted 100 µL of EPO assay diluent into each well.
3. Added 100 µL of standard, control or specimen per well. Gently tapped the plate frame approximately 1 min to mix well contents. Covered the plate with adhesive strip and incubated for 60 ± 5 min at room temperature on microplate shaker set at 500 ± 50 rpm.
4. Thoroughly aspirated or decanted the contents from each well. Blotted dry on clean paper towel.
5. Added 200 µL of EPO conjugate to each well. Covered the plate with a new adhesive strip and incubated for 60 ± 5 min at room temperature on microplate shaker set at 500 ± 50 rpm.
6. Aspirated each well and washed by filling each well with wash buffer (400 mL) using multi-channel pipette and completely removed liquid at each step, repeated the process three times. After the last wash, removed any remaining wash buffer by aspirating or decanting. Inverted the plate and blotted it against clean paper towel.

7. Added 200 µL of substrate solution to each well (Note: substrate solution must be used within 15 min of preparation) and incubated for 20-25 min at room temperature on the benchtop.

8. Added 100 µL of stop solution to each well. If color change did not appear uniform, gently tapped the plate to ensure thorough mixing.

9. Determined the optical density of each well within 15 min, using a microplate reader set at 450 nm and 620 nm (reference wavelength).

11.Data analysis

11.1 Measurements of pharmacokinetic parameters

Time to reach the maximal concentration (T_{max}, h)

Maximal plasma concentration (C_{max}, mIU/mL)

Elimination rate constant (K_e, h^{-1})

Plasma half-life ($t_{1/2}$, h)

Area under the concentration-time curve, 0-96 h (AUC_{0-t}, mIU·h/mL)

Area under the concentration-time curve, 0-∞ h ($AUC_{0-\infty}$, mIU·h/mL)

Clearance (CL/F, ml/h/kg)

Volume of distribution (Vd/F, L/kg)

Mean residence time (MRT, h)

11.2 Statistical analysis of pharmacokinetic parameters

The predose plasma concentration of endogenous erythropoietin ($C_{predose}$), C_{max} and T_{max} were obtained directly by visual inspection of the plasma concentration-time profiles. The slope of the terminal log-linear portion of the concentration-time profile was determined by least-squares regression analysis and used as the elimination rate constant (K_e). The elimination $t_{1/2}$ was calculated as $0.693/K_e$. The AUC_{0-t} from time zero to the last quantifiable point (Ct) was calculated using the linear trapezoidal method and extrapolation of AUC from Ct to infinity ($AUC_{t-\infty}$) was determined as Ct/K_e. Total $AUC_{0-\infty}$ was the sum of AUC_{0-t} + $AUC_{t-\infty}$. The total clearance (CL/F) was determined as the dose divided by the $AUC_{0-\infty}$. The mean residence time (MRT) was determined by statistical moment theory. All the PK

parameters were determined by non-compartmental analysis and the calculation was performed by using the TopFit, a pharmacokinetic data analysis program for PC. The mean relative bioavailability (F_{rel}) was calculated from $AUC_{0-\infty}$ ratio of Test/Reference. All pharmacokinetic parameters were described as apparent values since the ELISA cannot distinguish between endogenous and exogenous EPO.

The pharmacokinetic parameters of EPO were presented as mean ± SD. An analysis of variance (ANOVA) was used to determine the statistical differences of pharmacokinetic parameters (C_{max}, AUC) which represent the rate and extent of drug absorption. The variability between subjects, treatment groups, study period, and formulations were determined and the two one-side tests procedure were performed (64). Statistical analysis of AUC and C_{max} were calculated on logarithmically (ln) transformed data using ANOVA appropriate for the design. This approach generally assumes that the ln transformed data are distributed according to a normal distribution. Thereafter, using the variance estimate (S^2) obtained from the ANOVA, the 90% confidence interval (90% CI) for the ratio of AUC as well as C_{max} values of the test preparation over those of the reference products were estimated using the following computational formula:

$$90\%\ CI\ (\mu_T - \mu_R) = (\ \bar{X}_T\ -\ \bar{X}_R\ )\ \pm\ t^{v}_{0.1}\sqrt{\frac{2S^2}{n}}$$

where

$\bar{X}_T$, $\bar{X}_R$ were the observed means of the (ln) transformed parameters (either C_{max} or AUC) for the test product (T) and the reference (R)

S^2 was the variance estimate obtained from the ANOVA

n was the number of subjects

$t^{v}_{0.1}$ was the tabulated two-tail t value for 90% CI

v was the number of degree of freedom of the error mean square from the ANOVA

The antilogarithm of the 90% CI (μ_T - μ_R) would express a ratio of the test product and the reference product [μ_T/μ_R or Test/Reference]. The T_{max} difference was calculated as untransformed data (66, 67).