INTRODUCTION

Erythropoietin (EPO) is an endogenous glycoprotein hormone that regulates red blood cell production and proliferation from the bone marrow in response to tissue hypoxia and/or red cell mass reduction (1). EPO is primarily produced by a subset of peri-tubular endothelial capillary cells of the kidney and by some other tissues, including the brain, in response to hypoxia. The expression of EPO is controlled by a single gene on human chromosome 7 that produce a transcription factor or hypoxic-inducible factor (2-5). In adults, around 10% of the circulating EPO is produced by the liver (6). However, liver EPO has more important role during fetal development (7). In normal subjects, the range of circulating plasma EPO levels varies from 3.3-32.0 IU/L (1, 8, 9). Changes in plasma EPO concentrations are known to exhibit a circadian rhythm. The lowest EPO level is found at 8 AM., thereafter, the level increases by 40% at 4 PM and by 60% to the highest level at 8 PM (8).

In patients with end-stage renal disease (ESRD) or arthritis, the synthesis of EPO by the kidney is impaired and in patients with cancer or after chemo- or radiation therapy, the bone marrow does not respond effectively to EPO, leading to anemia. As a result, patients feel fatigue that, in the past, was treated with frequent blood transfusions, which only improved, but did not correct the anemia. Furthermore, anemia was found to be the risk factor for the development of left ventricular hypertrophy and heart failure that increased cardiac mortality in these patients (10, 11). Since the primary cause of anemia in these patients is an insufficient production of EPO (12) and blood transfusion is associated with a possibility of transmission of viral infection and many adverse reactions, treatment with exogenous EPO or recombinant human erythropoietin (rHuEPO) has been proved to be the most useful in decreasing blood transfusion requirements and increasing the quality of life in these patients (9). Moreover, rHuEPO can be used in the treatment of anemia in cancer, AIDS, chronic inflammatory diseases (13,14), rheumatoid arthritis (15) and diabetic patients (16).

In early 1950s, several investigators (17, 18) directly demonstrated the existence of a humoral erythropoietic factor by injecting large amounts of plasma from anemic animals into normal animals and observing an increase in blood reticulocytes or ^{59}Fe incorporation into new red cells, but it does not appear to affect white cell or platelet production. The initial work on purification of EPO was performed with plasma from anemic sheep in 1971 (19) and human EPO was purified in 1977, from 2,500 liters of urine from aplastic anemia patients (20). Human EPO was separated by sodium dodecyl sulfate (SDS) gel electrophoresis into two forms, native and asialo EPO (20). In 1985, the nucleotide sequence of the human EPO cDNA was reported and cloned in Chinese hamster ovary cells (CHO) that led to the production of large quantities of commercial rHuEPO (3, 21). Recombinant human erythropoietin was initially used for treating the anemia in patients with chronic renal failure in 1986, follow by the first pilot study in anemic cancer patients under chemotherapy in 1990 (22, 23). Other indications for rHuEPO therapy are anemia associated with autoimmune diseases, acquired immunodeficiency syndrome (AIDS), hepatitis C infection, congestive heart failure and in some surgical intervention (24). Furthermore, the role of rHuEPO as a survival factor for ischemic heart cells, brain cells and other organs is the focus of current research (25, 26).

The market for rHuEPO in Thailand is currently dominated by two innovator products, Eprex® (epoetin alpha) and Recormon® (epoetin beta). The two products are composed of the same amino acid sequence as endogenous EPO (165 amino acids), however, are varied in their glycosylation patterns. Glycosylation is a membrane-bound post-translational process which influences pharmacokinetics and affect their efficacy, safety, and immunogenicity. Nonetheless, there are no valid reports documenting any significant difference between epoetin alpha and beta with regards to the correction of renal anemia and adverse effects (27). Nowadays, these products are becoming the pre-eminent therapeutic biological agents and are one of the best-selling drugs in Thailand. Since the cost of the innovator products are relatively expensive, there has been a strong impetus to develop less expensive forms of these drugs. As patents covering the production and therapeutic use of epoetin alpha were expired, several pharmaceutical companies started developing what were then called 'biogenerics' or now referred to as 'biosimilar' rHuEPO.

The primary concerns for approval of biosimilar products are related to the manufacturing process since the products are manufactured using living organism. Biological products differ significantly from traditional drug products, which have low molecular mass and a known structure that facilitates generic copying. In contrast, biological products have relatively high molecular weight and much greater complexity than small-molecule drugs. Such products may exhibit significant heterogenicity in their molecular structures due to great variety factors, including the calls in which they are grow and the details of manufacturing process such as glycosylation and process-related impurities, which affect their pharmacokinetics, efficacy and safety. Under the European Medicine Agency (EMEA) biosimilar guidelines, biosimilar products must provide full chemistry, and demonstrated the preclinical and clinical data for approval as well as demonstrated efficacy and safety in pharmacovigilance after drug approval (28). The clinical studies composed of the pharmacokinetic study, pharmacodynamic study, clinical efficacy study, clinical safety study, pharmacovigilence and extension of indication studies. The pharmacokinetic study will compare the relative pharmacokinetic properties of the similar biological product with the reference product. This study should be determined in single dose crossover study using subcutaneous or intravenous administration. Healthy volunteers are considered an appropriate study population. The primary pharmacokinetic parameter is area under the concentration-time curve (AUC) and the secondary parameters are the maximal concentration (C_{max}) and half-life ($t_{1/2}$). But equivalence margins have to be justified primarily on clinical grounds. For the pharmacodynamic studies, reticulocyte count is a relevant pharmacodynamic marker for the activity of epoetin and recommended to be used in comparative pharmacodynamic studies. However, reticulocyte count is not an established surrogate marker for efficacy of epoetin and therefore no suitable endpoint in clinical trials. For clinical efficacy studies, equivalent therapeutic efficacy between the similar and the reference product should be demonstrated in at least two adequately powered, randomised, parallel group clinical trials. Confirmatory studies should preferably be double-blind to avoid bias. If this is not possible, at minimum the person(s) involved in decision-making (e.g. dose adjustment) should be blinded to treatment allocation. Sensitivity to the effects of epoetin is higher in erythropoietin-

deficient than non erythropoietin deficient conditions and is also dependent on the responsiveness of the bone marrow. Patients with renal anemia are therefore recommended as the target study population as this would provide the most sensitive model. For clinical safety study, safety data from at least 300 patients treated with the similar biological medicinal product in the efficacy trials is considered sufficient to provide an adequate pre-marketing safety database and to exclude excessive immunogenicity. The applicant should provide at least 12-month immunogenicity data in patients treated with the similar biological medicinal product. In this respect, retention samples for both 'titration' and 'maintenance' studies are recommended. For detection of anti-epoetin antibodies, a validated, highly sensitive assay should be used. In addition, the sponsor has to present a pharmacovigilance plan to address immunogenicity and potential rare serious adverse events. Special attention should be paid on the possibility of antibody-induced PRCA and immune-related adverse events.

The biosimilar product of epoetin alpha; Epokine® is approved for marketing in Thailand based on safety data from the preclinical studies in animals and clinical efficacy studies in end-stage renal disease (29, 30) and diabetic nephropathy (31), however, the comparative pharmacokinetic study between this product and the innovator is lacking. Therefore, this study was aimed to compare the pharmacokinetics of three formulations of 4,000 IU rHuEPO; epoietin alpha; Eprex®, Epokine® and epoietin beta; Recormon® after a single dose subcutaneous administration according to a randomized three-period crossover design to twelve healthy Thai male volunteers.

LITERATURE REVIEW

Recombinant human erythropoietin (rHuEPO)

Physicochemical properties

EPO is a glycoprotein of the cytokine family that folds into a compact globular form consisting of four alpha-helical bundles. Endogenous EPO has 165 amino acids with a molecular weight (MW) of 18,398 dalton (D). Its peptide core is responsible for EPO receptor-binding and suffices for *in vitro* stimulation of erythropoiesis, while the carbohydrate portion (40% of the total molecule) is important in the *in vivo* survival of EPOs (32). The overall MW of the EPO molecule is 30,400 D (30.4 kD) due to three nitrogen (N)-linked carbohydrate chains (at asparagines 24, 38 and 83) and one small oxygen (O)-linked oligosaccharide chain (at serine 126). Structural determination study revealed that the glycosylation sites are localized at one end of the molecule, distant to receptor binding site (33). Glycosylation occurs after translation, and structural differences of the four glycan residues result in numerous diverse isoforms of the molecule (34). The three N-linked carbohydrate chains may contain 2-4 oligosaccharide branches, each terminates with a negative charge sialic acid. The O-linked sugar chain carries up to two sialic acid residues. The total number of sialic acid residues determines the net negative charge of the molecule (27). Two internal disulphide linkages between cysteine residues 7 and 161, and 29 and 33 are known to be necessary for biological activity (13-16, 26, 27, 34, 35). While human urinary EPO and the rHuEPO are identical with respect to their primary and secondary structures, they exhibit minor differences in their composition of the N- and O-glycans. The functional consequences of the microheterogeneity of glycans of endogenous EPO are not well understood. However, the survival of circulating EPO relies on the presence of terminal sialic acid residues of N-glycans. Since galactose is the pre-terminal sugar of the glycans, de-sialylated EPO is rapidly cleared in the liver via galactosyl receptors of the hepatocytes (36). Endogenous EPO and the epoetins contain up to 14 sialic acid

residues per molecule. The epoetins are probably glycosylated more completely than endogenous EPO, as the specific *in vivo* biological activity of rHuEPO is greater (approximately 200,000 IU/mg peptide) than that of purified human urinary EPO (70,000 IU/mg peptide) (37).

The conventional rHuEPO preparations, epoetin alpha and epoetin beta, are engineered in CHO cell cultures that were originally transfected with the authentic human EPO gene. Epoetin alpha and epoetin beta differ in size and charge. Epoetin beta has higher MW, contains a wider spectrum of isoforms, and has a higher proportion of more basic isoforms, consistent with a lower number of sialylated glycan residues than epoetin alpha (27, 38). Although, the proportion of tetra-sialylated carbohydrate residues of epoetin beta is highest in comparison with other epoetins, but, there are no reports that epoetin alpha differs from epoetin beta in its clinical efficacy.

Mechanism of action

EPO trans-membranous receptors (EPO-R) belong to the cytokine receptor family. This receptor family has tertiary structure comprises receptors for EPO, various interleukins, granulocyte macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), growth hormone and prolactin. The special characteristic of this receptor family is that it switchs on and transduces signal to the interior of cell (39, 40).

EPO-R are mainly expressed by the erythroid progenitors, burst-forming units-erythroid (BFU-E) and colony-forming units-erythriod (CFU-E) in bone marrow. EPO can stimulate BFU-E and CFU-E, for their proliferation and differentiation (39). EPO-R are a homodimer of two glycoprotein chains of 484 amino acids. The two EPO-R sub-units usually share one EPO molecule, whereby the dissociation constants for the two binding sites differ greatly (1 μM vs 1 nM). EPO binding induces a conformational change and tighter connection of the EPO-R dimers. As a result, two Janus kinase 2 (Jak2) tyrosine kinases are activated and come into contact with the cytoplasmic region of the EPO-R molecules. The phosphorylated tyrosine residues of EPO-R act as docking sites for several signaling proteins. In turn, various kinases, anti-apoptotic protein and transcription factor are activated (Figure 1). The rapidity of

this process is the primary event in signal transduction (35, 37, 41, 42). The action of EPO is mediated through mRNA and subsequent protein synthesis, rather than DNA replication and cell division (43). After EPO stimulation, rapid increases in erythrocyte specific mRNA and protein are observed. The viability and maturation of erythroid progenitor cells is greater in the presence of EPO (35, 44).

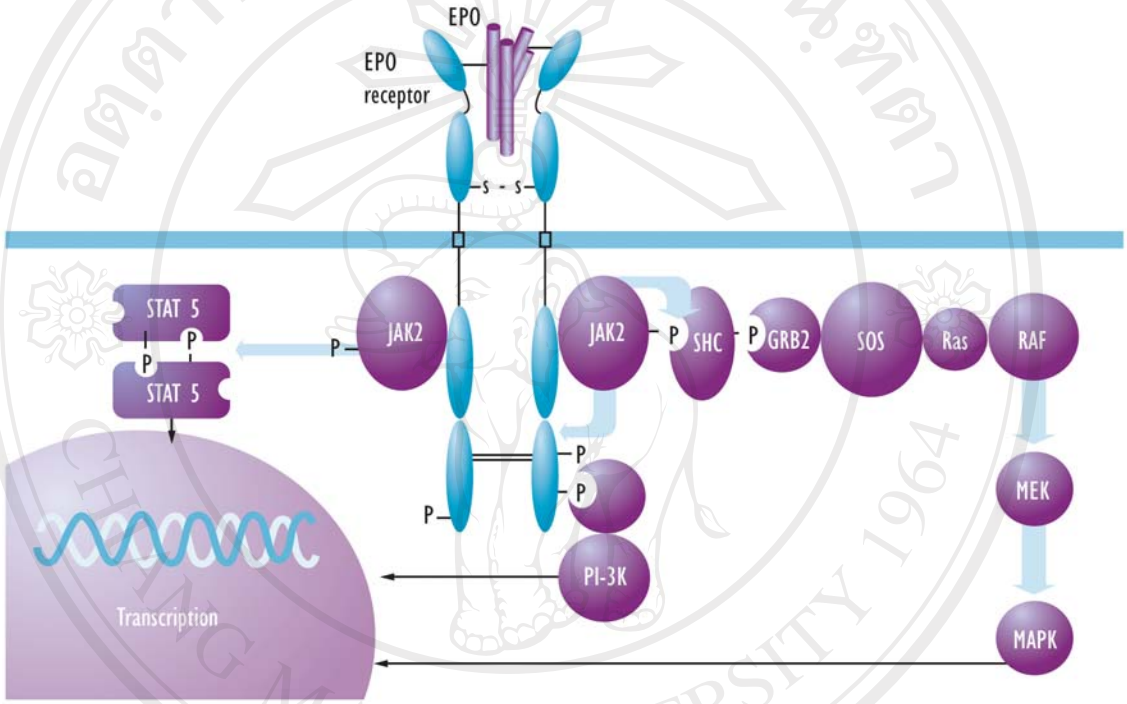

Figure 1 Effect of the binding of EPO to EPO receptor.

EPO signaling involves autophosphorylation of Janus kinase 2 (JAK2), phosphorylation of EPO receptor (EPO-R), homodimerization of signal transducer and activator of transcription 5 (STAT5), activation of phosphatidyl-inositol-3-kinase (PI-3K), phosphorylation of the adapter protein SrC-homology and collagen (SHC) to form a complex with growth factor receptor binding protein (GRB), son of sevenless (SOS) and the G-protein Ras, and the sequential activation of the serine-kinase Raf, mitogen-acivated protein kinase kinase (MEK or MAPKK) and mitogen-activated protein kinase (MAPK). The signaling cascade results in survival, proliferation and differentiation of erythrocytic progenitors. Following de-phosphorylation, the EPO/EPO-R complex is internalized and degraded (37).

A study of the kinetics of the physical interaction between EPO and EPO-R has shown that the carbohydrate of EPO prevents binding to EPO-R through electrostatic forces (45). The affinity of EPO to EPO-R decreases with an increased number and complexity of N-glycans. Non-glycosylated rHuEPO has a 10-fold higher receptor binding activity than glycosylated rHuEPO (46).

Pharmacokinetics properties

Absorption and Distribution

After subcutaneous administration, EPO is absorbed slowly and serum EPO concentrations are lower than after intravenous (i.v.) administration but are maintained for several hours (47). The bioavailabilities of epoetin alpha (30-36%) and epoetin beta (15-50%) after subcutaneous administration are lower than those after i.v. administration (27).

Metabolism and excretion

Clearance of EPO appears to be via three potential routes. These include, excretion through the kidney, metabolism by the liver, and consumption by the erythron (35). The action of EPO is terminated by the haematopoietic cell phosphate (HCP or SHP1). On de-phosphorylation, the EPO/EPO-R complex is internalized and degraded. It is assumed that EPO-R-mediated uptake of EPO by erythropoietic tissues is the major mechanism of the removal of EPO from circulation (48). *In vitro* studies show that internalization of the EPO/EPO-R complex by the target cells is rapidly followed by its proteasomal and lysosomal proteolysis (49). Also the proteasome is responsible for the down regulation of the EPO-R in EPO-stimulated cells, as it prevents the appearance of newly synthesised EPO-R (50). The apparent paradox, that glycosylated EPO with reduced EPO-R binding activity have strong *in vivo* activity, can be explained by the counteracting effect of sialic acid containing carbohydrate chain on the rate of EPO clearance (46). EPO is present in urine, however, the contribution of the kidney to clearance is less than 10% (51). The liver rapidly metabolizes desialated EPO, and the removal of the sialic acid residues of EPO abrogates its bioactivity *in vivo* but not *in vitro* (52, 53).

The dose-sparing effect has been attributed to the extended half-life after subcutaneous administration resulting in a sustained stimulation of erythroid progenitor cells. Half-lives for epoetin alpha and beta after i.v. vs subcutaneous routes are 4-11 h vs 19-25.3 h and 8.8-10.4 h vs 24 h, respectively (27).

Dosage and administration

In the treatment of anemia in chronic renal failure patients, subcutaneous administration is preferred over the i.v. administration because the absorption is slower and the amount of drug required is reduced by 20% to 40% (52). Patients are started on dose of 80 to 120 IU/kg of EPO, subcutaneously three times a week. It can be given as once-a-week schedule, but somewhat more drug is required for an equivalent effect. If the response is poor, the dose should be progressively increased. The final maintenance dose of EPO can vary from as little as 10 IU/kg to more than 300 IU/kg (average 75 IU/kg), three times a week. The target hemoglobin (Hb) should be greater than 11 g/dL according to the European Best Practice Guidelines (EBPG) and the US-based National Kidney Foundation's Kidney Disease Outcome Quality Initiative (K/DOQI) (54, 55). Hb should not be raised by rHuEPO therapy into the normal range, particularly not in patients with cardiovascular disease. In general, brain and heart function, physical endurance capacity and quality of life parameters are sufficient if Hb levels are maintained in the range of 11-12 g/dL or hematocrit (Hct) 33% to 36% (37). The dose of EPO should be adjusted to obtain a rise in the Hct over a 2- to 4-month period to a final Hct of 33% to 36%. An increase in reticulocyte count is usually observed in about 10 days whereas an increase in hemoglobin and hematocrit levels are observed in 2–6 weeks. Patients with endogenous EPO level less than 100 IU/kg have the best chance of response whereas those with endogenous EPO between 100-500 IU/kg respond occasionally. Failure to respond to EPO is most commonly due to concurrent iron deficiency. Prior to initiation of therapy, the patient's iron stores should be evaluated. Transferrin saturation and ferritin should be at least 20% and 100 ng/mL, respectively. Folic acid and vitamin B12 deficiencies should be ruled out before EPO administration, and serum potassium levels, blood pressure and platelet counts should be monitored as well (56).

Drug interactions

No evidence exists to indicate that treatment with EPO alters the metabolism of other drugs. However, since cyclosporine is bound by red blood cells, there is a potential for drug interaction. If EPO is given concomitantly with cyclosporine, blood levels of cyclosporine should be monitored. The effect of EPO may be potentiated by the simultaneous therapeutic administration of a haematinic agent, such as ferrous sulphate, when a deficiency state exists. Drugs that decrease erythropoiesis may decrease the response to EPO (57).

Adverse effects

rHuEPO is generally well tolerated. The adverse events reported are often associated with the complication of chronic renal failure. Headache, hypertension and seizures have been seen in patients with poor renal function. The most important of which is hypertension which can lead to encephalopathy and seizures (58). These adverse effects are probably associated with too rapid rise in hematocrit. Blood pressure should be controlled by increasing antihypertensive therapy. Low dose EPO therapy and the more gentle attainment of target hemoglobin levels have largely overcome this phenomenon. Subcutaneous injection of EPO has therefore been proved to be the most satisfactory route for administration (59). Treatment to attain hematocrit level greater than 36% is not recommended (60). Study of patients treated to raise hematocrit above 40% shows a higher incidence of myocardial infarction and death (61). An increased tendency to vascular thrombosis in dialysis patients also has been reported as well as transient increase in platelet count, flu-like symptoms, hyperkalemia, and skin rashes. Subcutaneous administration of EPO may cause redness, swelling or itching at the site of injection. Skin rashes and urticaria have been observed rarely and when reported have generally been mild and transient in nature. There have been rare reports of potentially serious allergic reaction including urticaria associated with respiratory symptoms or circumoral edema or urticaria alone with previous formulation of epoetin that contained citrate-buffered protein solution and gelatin hydrolysate (62). To eliminate the risks of anaphylaxis, the new formulations of phosphate-buffered epoetin that do not contain gelatin hydrolysate are now being used. Pure red cell aplasia (PRCA) associated with neutralizing antibodies

to endogenous EPO and rHuEPO have been reported in chronic renal failure patients with long term use of rHuEPO. The majority of cases have been observed after subcutaneous administration of epoetin alpha after removing of human albumin serum from the formulations (63, 64).

Precautions and contraindications

Blood pressure in all patients receiving EPO should be closely monitored and adequately controlled as necessary prior to the initiation of EPO therapy. If blood pressure cannot be controlled, EPO treatment should be discontinued. EPO should be used with caution in patients with refractory anemia with excess blasts, a history of seizures, epilepsy and chronic liver failure. Patients with condition associated with thrombotic/vascular events should be closely monitored. Contraindication is dictated in patients with uncontrolled hypertension, hypersensitivity to active substance or to any of excipients, and in surgery patients who for any reason cannot receive adequate antithrombotic treatment (43, 44).

OBJECTIVES

The purposes of this study was to compare the pharmacokinetics of three formulations of 4,000 IU recombinant human erythropoietin; epoietin alpha (Eprex®), a biosimilar product of epoietin alpha (Epokine®) and epoietin beta (Recormon®) after a single dose subcutaneous administration in healthy Thai male volunteers.