# I. INTRODUCTION

Erythropoietin (EPO) is a 30,4-kDa glycoprotein hormone produced in the liver and kidney that enhances the growth of erythrocytic progenitors in bone marrow (Melmed et al., 2020) and plays a key regulatory role in erythrocyte production (Suresh et al., 2020). EPO is primarily produced from renal EPO-producing cells, or REPs, and it is tightly controlled in a hypoxia-inducible manner to maintain tissue oxygen homeostasis (Souma et al., 2015). In 1977, human EPO was first discovered in the urine of anemic patients; its gene was then discovered in 1983 (Kalantar-Zadeh, 2017). In recent years, EPO has been successfully cloned in Chinese Hamster Ovary (CHO) cells and currently, recombinant human erythropoietin (rHuEPO), developed from EPO is commonly used for anemia medication. In 1989, Amgen produced the first rHuEPO, epoetin alfa, which was marketed as Epogen® and distributed to dialysis patients in the United States (Kalantar-Zadeh, 2017). Other businesses, including Johnson & Johnson and Ortho Biotech, eventually began producing and selling EPO products as well. Erythropoietin stimulating agents (ESAs) have been used to treat anemia in a variety of patients since the discovery of erythropoietin, including those with cancer, chronic kidney disease (CKD), hepatitis C infection, cardiovascular disease, and human immunodeficiency virus infection, where anemia is a common issue (Aapro et al., 2019).

The production of EPO combined with human serum albumin (HSA) was used to treat patients before 2004 but it later became a concern because human serum albumin (HSA) might promote a variety of Creutzfeldt-Jakob diseases (CJD). It became challenging to obtain regulatory approval for medications having components of mammalian origin in the 1990s because it was discovered that CJD might be transmitted from diseased cows (Sønderby et al., 2018). Creutzfeldt-Jakob disease (CJD) is a rare, deadly degenerative brain disorder characterized by prion proteins and classified as a transmissible spongiform encephalopathy that causes fast progressing dementia (KNOPMAN, 2004; Marques et al., 2018). Polysorbate 80 was substituted for human serum albumin (HSA) as the stabilizer in Eprex in 1998 because of the CJD issue (McKoy et al., 2008). Polysorbate 80 (PS80) is a surfactant that is often employed in therapeutic protein formulations to reduce adsorption and interface-induced protein aggregation (Grabarek et al., 2020). HSA was also replaced with polysorbate 80 to get a halal logo for the Indonesian market because 86.7% of Indonesians are Muslims, therefore the polysorbate became the best option to be used in the product.

The efficient stabilization of erythropoietin by HSA was found to be superior to that by polysorbate 80, as a result of the formulation modification and inappropriate handling of less stable products would be more likely to induce aggregation (Macdougall et al., 2012). There are several circumstances of treatment using EPO with Polysorbate 80 that have shown complicacy during the EPO treatment period, such as pure red cell aplasia (PRCA), which is defined as anemia brought on by the failure of erythropoiesis (Sloand et al., 2006). Patients receiving treatment with ESA may develop pure red cell aplasia (PRCA), an extremely uncommon but severe anemia that requires transfusions (Shingu et al., 2020). The symptoms of epoetin-associated pure red cell aplasia (PRCA) include severe anemia, a low reticulocyte count, the absence of erythroblasts, epoetin nonresponse, and neutralizing antibodies against Erythropoietin (EPO) (McKoy et al., 2008). There are two main reasons that could be the main cause of PRCA caused by EPO, such as the leachates of organic compounds caused by rubber stoppers used in the product packaging and the occurrence of aggregate inside the product caused by storage and mishandling of the product.

The syringe for the product packaging uses coated rubber stopper to eliminate any chance of organic contaminant from the contact of rubber stopper with the product (Boven et al., 2005). PT. Kalbio Global Medika has solved this problem by coating the rubber stopper using silicone oil. The coated rubber stopper decreases the chance of the organic substance to intermix with the drug product and cause aggregate to occur. Due to its low cost and environmental friendliness, silicone

oil as rubber stopper coating has received a lot of interest (Zhu et al., 2017). Since silicone oils naturally have very low coefficients of friction, they have been utilized for many years as excellent lubricants for polymers and rubbers (Abdelbary, 2014). The other reason for rubber stoppers coated with silicone oil is to prevent sticking when placed in feeder bowls that are used to transfer goods to the main packaging container (Rothhaar et al., 2022). Since PT Kalbio Global Medika has solved the leachate problem from rubber stoppers by silicone oil coating, the remaining possibility that may cause PRCA comes from the aggregate formation of EPO protein is the storage and mishandling of the product. Therefore, it is important for PT Kalbio Global Medika to quantify the polysorbate EPO aggregate formation. The aggregation of EPO in PT Kalbio Global Medika drug product was performed by heating treatment (force degradation treatment).

The method used in PT. Kalbio Global Medika to detect aggregate is by using liquid chromatography. There are two types of liquid chromatography available at PT. Kalbio Global Medika, which are High Performance Liquid Chromatography (HPLC) and Ultra Performance Liquid Chromatography (UPLC). The most efficient liquid chromatography known to be UPLC because of its sensitivity to detect smaller proteins, shorter running time, and small amount of sample required for the trial because of the higher pressure that is available in UPLC. UPLC is also used when the particle size of the sample is smaller than 2 µm while for HPLC it ranges from 3 µm to 10 µm (Kumar T et al., 2020). UPLC and HPLC have different types of columns. The column used in UPLC can not be used for HPLC because the UPLC column has smaller particles and narrower diameter because the pressure in UPLC is higher than HPLC.

The UPLC instrument will use the reverse phase (RP) column, while the HPLC instrument will use the size exclusion (SEC) column. The instruments did not use the same column because of the unavailability of the same type of column for the instruments at PT. Kalbio Global Medika. The reversed-phase UPLC method that separates molecules based on hydrophobicity and the size exclusion HPLC that separates molecules based on their size will be used in this project because EPO proteins are suspected to have different hydrophobicity from aggregates. The result will be observed from the chromatography of the EPO and aggregate peak area with %area to calculate the aggregate percentage in EPO. The limit of quantification will also be calculated to determine the lowest percentage of aggregate that can still be detected by the UV detector. The recovery and the limit of quantification (LOQ) will be determined by observing the result after the pre-validation of the method which will be carried out by testing linearity, precision, and accuracy.

The hypothesis of the result is that the peak of the aggregate is separated from the erythropoietin peak in the chromatogram. The aim of this project is to observe the implication of RP-UPLC and SEC-HPLC in detecting and quantifying polysorbate EPO aggregate peak and to determine which liquid chromatography method was better in detecting and quantifying polysorbate EPO aggregate.

This paper reports the sensitivity of RP-UPLC and SEC-HPLC methods in detecting the aggregate of EPO. The most acceptable method in detecting the aggregate of EPO will be further used in pre-validation to check whether the method can be used in the future to detect EPO aggregate. To the best of our knowledge, no method was performed in detecting the molecular weight of EPO aggregate.