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Method Development and Method Validation for Residual Water Determination of Erythropoietin Pharmaceutical Formulations by Volumetric Karl Fischer Titration

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Abstract

The aim of this study is to develop and validate a water determination method for erythropoietin products using volumetric Karl Fischer (KF) titration technique. The external extraction technique using Hydranal™ formamide dry as an extraction solvent and using a mixture solution of Hydranal™ methanol dry and Hydranal™ formamide dry in a ratio of 60:40 as a solvent in the titration vessel was designed as a suitable method. The developed method was validated for specificity, repeatability, accuracy, and intermediate precision. The method was specific to the residual water contained in the erythropoietin pharmaceutical products as there was no effects from any interferences. Repeatability expressed the relative standard deviation (RSD) of six replicate samples as 4.6% (≤10%), which clearly revealed that the method was precise. The % recoveries obtained from three concentration points (lowest %residual water content, 100% and 120% of specification) were 101% , 104% and 105%, which were within the acceptable range of 90-110% . It obviously indicated that the method provided a very acceptable accuracy. The intermediate precision outcomes on different days and between analysts were shown in terms of % margin. % Margin was observed as an excellent value of 6% (≤25%). This indicated that the method was reproducible. In conclusion, it was indicated that the developed method was suitable for water determination in erythropoietin products since the validation results show that the method was specific, precise, accurate, and reproducible.

Keywords: Erythropoietin, lyophilized powder, method validation, residual water determination, volumetric Karl Fischer titration

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Introduction

Erythropoietin is manufactured by recombinant DNA technology in cell culture (Ifeanyl, 2015). The produced erythropoietin manifests the identical structure as the human erythropoietin, which composed of 165 amino-acid glycoproteins with molecular weight of 30,400 Daltons (Varas et al., 2018). Furthermore, the recombinant human erythropoietin also exhibits the equivalent activity as the endogenous erythropoietin which acts as a stimulator of red blood cell production in the bone marrow (Ifeanyl, 2015; Grampp et al., 2018). Erythropoietin is indicated for treatment of anemia in both adults and pediatric patients with various associate conditions due to chronic kidney disease, anti HIV infection and myelosuppressive chemotherapy (Bohlius et al., 2006; Henry et al., 1992; John et al., 2012). The erythropoietin pharmaceutical formulations in the market are available in two dosage forms; concentrated solution and lyophilized powder (Veiga et al., 2013). Among 44 erythropoietin products officially registered by Thai FDA, 10 formulations are lyophilized powder (Thai FDA, 2019).

Lyophilization is the process of removing water presented in the products under low temperature and pressure (Joseph E, 2019). Residual water in lyophilized drug products has a critical impact on stability of the active ingredient, particularly large molecules such as proteins (Joseph E, 2019). Increasing residual water content causes degradation of products via hydrolysis reaction. It results in not only physico-chemical stability (e.g., enhancing collapse of porous structure and altering reconstitution behavior), but also biological stability which can affect biological activity (Joseph E, 2019). Therefore, the content of residual water in finished products should be in a range of 2-3% (Chang et al., 2005). The residual water contained in the products mainly achieves from the inactive ingredients in the formulation. Amount of 4,000 international units (IU) of erythropoietin corresponded to 26.68 µg, which was less than 0.05% w/w in the formulation (Kato T, 2016). Owing to the hygroscopic property, lyophilized erythropoietin products easily adsorb moisture from the atmosphere leading to a false high value in water content (Joseph E, 2019). Therefore, an appropriate method to accurately and precisely determine residual water content of these products needs to be developed.

Karl Fischer (KF) titration is the recommended technique for water determination in the official pharmacopoeias such as British Pharmacopoeia and United States Pharmacopeia (British Pharmacopoeia, 2019; USP, 2019). Volumetric KF titration is preferred to measure the water content in the range of 0.01% to 100% or between 0.1 mg and 500 mg of water (Margreth et al., 2010). In general, water extraction from samples can carry out in two dissimilar ways; internal and external extraction (Margreth et al., 2010; Mettler Toledo, 2011). Regarding the internal extraction, the sample powder is directly transferred into the titration vessel and then the residual water is extracted out and titrated (Margreth et al., 2010; Mettler Toledo, 2011). On the other hand, the external extraction requires to dissolve the sample powder with a proper solvent prior to transferring into the titration vessel (Margreth et al., 2010; Mettler Toledo, 2011). The water content of the solvent used has to be determined and subtracted from the gross water content to provide the actual water content of the sample. As the concern of hygroscopic property, external extraction technique is interesting. A suitable solvent for complete water extraction therefore, is necessary to be investigated since the excess of some kinds of the solvents affect the stoichiometry of the KF reaction (Margreth et al., 2010; Mettler Toledo, 2011). The accomplishment of titration on solvent effect must consequently be evaluated as well.

In our experiment, we performed volumetric KF titration with external extraction to determine the residual water content of erythropoietin products. The objective of this study was to develop and validate an analytical method to determine the residual water content of erythropoietin

in the lyophilized products using the external water extraction and volumetric KF titration. We examined the ability and potential of this method to determine the amount of water in lyophilized erythropoietin products in terms of the resulting products' quality, efficacy and stability.

Materials and Methods

Chemicals and reagents

Two different organic solvents: Hydranal™ methanol dry and Hydranal™ formamide dry were purchased from Honeywell, Germany. Hydranal™ Composite 2 (KF reagent) and Water standard 10.0 (1%w/w) were also bought from Honeywell, Germany. Mannitol and human serum albumin (HSA) were acquired from Sigma Aldrich, USA. Sodium chloride, sodium phosphate monobasic monohydrate and sodium phosphate dibasic dihydrate were obtained from Carlo Erba, USA. Recombinant human erythropoietin products were received from the distributors.

Apparatus

Residual water content measurements were performed using a volumetric KF titrator (Metrohm Ltd., Switzerland) equipped with a 5-mL exchange unit. Other equipment and supplies employed included an analytical balance (Mettler, Switzerland), a hot air oven (Memmert, Germany), and syringes with needles (Nipro, Thailand).

Methods

Preparation of lyophilized matrix sample

Components of the matrix solution represent all excipients contained in the erythropoietin products marketed in Thailand. The matrix solution in the presence of mannitol, human serum albumin, sodium chloride, sodium phosphate monobasic monohydrate and sodium phosphate dibasic dihydrate were prepared in sterile water for injection. One milliliter of matrix solution was filled in each 5-mL glass vial and approached to the lyophilization process. The lyophilized matrix samples were stored in a desiccator at 2-8^{o}C.

Method development

Selection of external extraction solvent

Anhydrous methanol and anhydrous formamide are the desired solvent considered to dissolve lyophilized samples (Joseph E, 2019; Mettler Toledo, 2011). Thus, these two solvents were chosen as the preliminary solvents for studying the reconstitution of the lyophilized matrix samples and erythropoietin products. We evaluated the solubility of the lyophilized matrix sample in two different external extraction solvents; Hydranal™ methanol dry and Hydranal™ formamide dry. A vial of lyophilized matrix sample was reconstituted with each extraction solvent. The suitable solvent selected was then used to reconstitute the lyophilized erythropoietin products to examine its ability to act as an extraction solvent. If the reconstitution was successful, then the solution had a clear appearance.

Table 1. The study design for determination of the suitable external extraction solvent

Sample	External extraction solvent
Lyophilized matrix sample	Hydranal™ methanol dry Hydranal™ formamide dry
Erythropoietin product	Suitable solvent obtained from dissolving lyophilized matrix samples

Selection of solvent in titration vessel

We compared two different solvents, Hydranal™ methanol dry and a mixture of Hydranal™ methanol dry and Hydranal™ formamide dry in the ratio of 60:40, for use as the solvent in the titration vessel. Titration of each of 2-mL reconstituted matrix sample in the titration vessel containing each solvent studied was performed. The stoichiometry of the KF reaction indicated by the constant drift values of the titration using different solvent in the titration vessel was evaluated.

Determination of residual water content of external extraction solvent

In our method development process, first we found the suitable external extraction solvent for reconstituting the lyophilized sample. Next, we determined the residual water content of the selected extraction solvent for external extraction prior to assessment of the validation parameters. We subtracted the water content of the extraction solvent from the residual water content of the reconstituted samples to calculate the actual residual water content of the samples .

Approximately 2 mL of the extraction solvent was drawn into a syringe and the whole syringe was then accurately weighed. The solvent was quickly transferred into the titration vessel and the syringe with the remaining solvent was re-weighed. Residual water contained in the extraction solvent was titrated until reaching an end point. An average value of the residual water content of the solvent was obtained from triplicate measurements.

Method validation

Five validation parameters with respect to specificity, repeatability, accuracy and intermediate precision were utilized to assess the developed method.

Specificity

Interferences of air in the titration system and KF reagent on the measurement results were studied. Two samples, air and 0.5 mL of Water standard 10.0, were used as the samples for this study. The influence of air in the titration system was also evaluated by performing the titration with no sample added into the solvent in the titration vessel. The effect of KF reagent was evaluated by the titration of the known amount of water content contained in the Water standard 10.0. We directly quantified the water content of the air in the titration system was. We calculated the percent recovery of the residual water contained in the Water standard 10.0 as follows:

$$\%\text{Recovery} = \frac{\text{Water content found in Water standard 10.0}}{\text{Water content stated in the certificate of Water standard 10.0}} \text{ x } 100 \qquad \text{(Equation 1)}$$

Repeatability

Six lyophilized matrix samples were left at ambient temperature for 30 minutes. A whole vial of the matrix sample including the lyophilized powder, a glass vial, a rubber stopper, and an aluminum cap were weighed. The lyophilized matrix sample was completely reconstituted with 2 mL of the suitable extraction solvent. The residual water in the lyophilized matrix sample was extracted by gently mixing until a clear solution was obtained. We used a syringe with a needle to transfer the matrix solution from the vial to the titration vessel. Then we titrated the solution. The vial with remaining solution, the rubber stopper, and the cap were dried in an oven at 230°C. Then we cooled down the vial to the ambient temperature in a desiccator before re-weighing. The weight of the vial after drying was subtracted from the weight of the whole vial to calculate the weight of the remaining matrix sample. Percent residual water content of the lyophilized matrix sample was calculated. Six replicate determinations of % residual water content were performed. Then we calculated the % RSD. The average % residual water content obtained was assumed to be the lowest water content determined in the lyophilized matrix samples.

Accuracy

Accuracy testing was carried out by spiking the water standard 10.0 in the matrix solution at three concentration levels. Three levels were comprised of lowest %residual water content (obtained from repeatability), 100% and 120% of the specification (specification of the residual water content allowed to contain in the erythropoietin pharmaceutical formulations: not more than 4%) which covered the working range. Three replicate determinations of each level were carried out. The % recovery obtained from a comparison of water content found and water content added was calculated as follows:

$$\%\text{Recovery} = \frac{\text{Water content found}}{\text{Water content added}} \text{ x } 100 \qquad \text{(Equation 2)}$$

Intermediate precision

Intermediate precision was presented to measure within-laboratory variations resulting from performing the procedure being conducted by three different analysts on three different days. Three individual vials of the erythropoietin product were determined on each three different days by the major analyst. Also, the other 6 individual vials (3 each) of the erythropoietin product were demonstrated by the other two second analysts. The average value of the % residual water content and standard deviation (SD) of three different days and three different analysts were calculated. The following % margin was used for evaluation of the intermediate precision studies.

$$\%\text{Margin} = \frac{\text{SD in intermediate precision x } 2.575}{\text{Upper specification limit} - \text{Mean}} \text{ x } 100 \qquad \text{(Equation 3)}$$

Results and Discussion

Method development

Selection of external extraction solvent

The results show that the Hydranal™ formamide dry was superior to the Hydranal™ methanol dry in terms of the dissolving of lyophilized samples. Figure 1(a1) shows that the lyophilized matrix sample did not dissolve with the Hydranal™ methanol dry. A turbid suspension was obviously observed after reconstitution. This was associated with the prior reports, which explained that mannitol and human serum albumin were not able to dissolve in alcohol (Bouchard et al., 2007; Houen G, 1996; Yoshikawa et al., 2012). On the contrary, the lyophilized matrix sample was much soluble in the Hydranal™ formamide dry (Figure 1(a2)). Therefore, the Hydranal™ formamide dry was selected for the further step. The erythropoietin product was reconstituted with the Hydranal™ formamide dry. The clear solution obtained (Figure 1(b)) is evidence that the erythropoeietin product was completely reconstituted with Hydranal™ formamide dry. It was concluded that Hydranal™ formamide dry was the suitable solvent for using as the external extraction solvent for the lyophilized matrix sample and erythropoietin products.

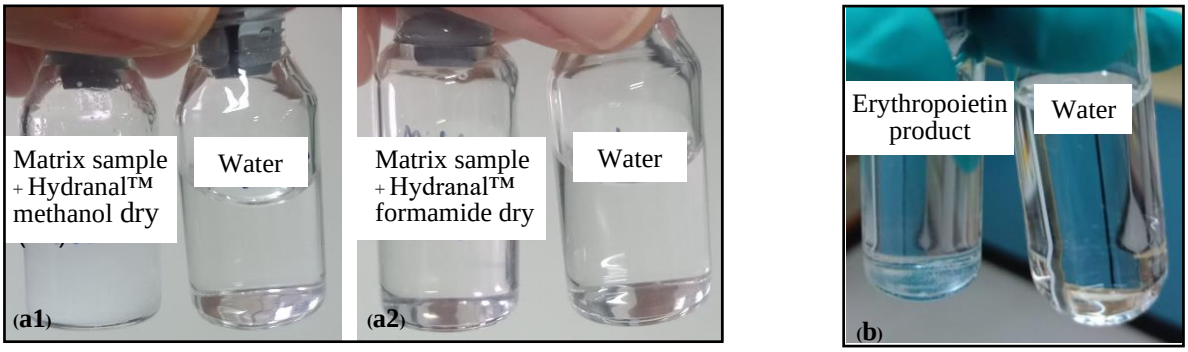


Figure 1. Clarity of the reconstituted solutions (a) lyophilized matrix samples reconstituted with the Hydranal™ methanol dry (1) and with the Hydranal™ formamide dry (2) and (b) erythropoietin product reconstituted with the Hydranal™ formamide dry

Selection of solvent in titration vessel

In the preliminary study, we noticed that using Hydranal™ methanol dry as a solvent in the titration vessel resulted in the precipitation of mannitol and HSA. This led to speedily reach the end point. It can be explained that the precipitates coated on surface of the electrode and blocked the titration reaction (Bouchard et al., 2007; Houen G, 1996; Yoshikawa et al., 2012). The water was not able to react with the KF reagent and the end point then was reached very fast resulting in incorrect volume of KF reagent consumed. Besides, the constant drift value, which is the value indicated the water amount entering to the titration system per unit time, reached to 0 uL/min (Margreth et al., 2010; Mettler Toledo, 2011). This extremely low value of the constant drift caused by the completely blockage of the precipitation of mannitol and HSA on the surface of the electrode. To overcome this challenge, the other solvents were required to improve the solubility of mannitol and HSA. Thus, we found that using of a mixture of Hydranal™ methanol dry and Hydranal™ formamide dry in the ratio of 60:40 as a solvent in the titration vessel did not affect the constant drift values and the end point. The mixture of solvents seemed solve the precipitation problem because the excipients

in matrix sample dissolved in this mixture (Margreth et al., 2010; Mettler Toledo, 2011). The result was in agreement with the literature reported that the addition of formamide (not more than 50%v/v) in the titration system improved the stoichiometry of the KF reaction (Margreth et al., 2010). The Hydranal™ formamide dry in the ratio of 40% of mixture solvent was designed in order to have some percent available of the Hydranal™ formamide dry in the system for the reconstituted samples added. Hence, the mixture of Hydranal™ methanol dry and Hydranal™ formamide dry in the ratio of 60:40 was proper to use as the solvent in the titration vessel.

Determination of residual water content of external extraction solvent

Even though the Hydranal™ formamide dry used as the extraction solvent is identified as "dry", the solvent still contained a small amount of water. This residual water could not be ignored, since it could affect the analysis of residual water of the sample. Therefore, we determined the residual water content of the extraction solvent before analyzing the sample. The residual water content of the extraction solvent was subtracted from residual water content of the reconstituted sample in order to calculate the true amount of residual water in the sample.

Method validation

Specificity

Firs, we evaluated whether aspects of the laboratory environment affected the titration system by titrating the air in the system. We found that air in the system contained only 0.077 mg water. This amount of water was insignificant since the detection limit of volumetric KF titration method was 0.1 mg water (Margreth et al., 2010). Consequently, water in environment of the titration system did not have an effect on the experiments and could be safely ignored. Next, Water standard 10.0 was titrated to ensure that there was no interference from the KF reagent. The recovery of the known amount of water content in the Water standard 10.0 was 100.6%. This % recovery was within the acceptance criteria of 97.5-102.5% (British Pharmacopoeia, 2019). The high accuracy for the %recovery indicated that KF reagent did not interfere with the titration system. We also concluded that the method was specific to only water.

Repeatability

Six individual lyophilized matrix samples were analyzed to determine repeatability. Average residual water content in the lyophilized matrix samples shown as 3.8% water and the %RSD was 4.6%. The results complied with the acceptance limits of 4.0% water (British Pharmacopoeia, 2019) and 10.0%RSD respectively (Incledon & Lam, 2004). Thus, we concluded that the method was repeatable for residual water content determination in the analyzed samples.

Accuracy

The accuracy data was presented in Table 2. The %recovery of the three concentration points (lowest %residual water content, 100% and 120% of the specification) was 101%, 104% and 105%, respectively. These %recovery values were all within the acceptance limits between 90% to 110% which was indicated that the water content found were very close to the true water content containing in the test solution (OECD, 2014). In conclusion, the method was able to determine the residual water content accurately.

Table 2. %Recovery of the accuracy study

Concentration of water content	%Recovery
3.8%	101%
4.0%	104%
4.8%	105%

Intermediate precision

Intermediate precision was performed by measuring residual water content in the erythropoietin product on the three different days and by the three different analysts. Erythropoietin product was used as the test samples in this study in order to ensure the usability of the developed method after the specificity, repeatability and accuracy of the method was satisfactorily validated. Table 3 shows the results of the intermediate precision. The average residual water content in the products on the three different days by the main analyst were 0.5%, 0.7%, and 0.5% , respectively. While the average residual water content in the products determined by the other two second analysts were 0.7 and 0. 5% , respectively. The results were excessively low with respect to the acceptance limit of 4. 0% water (British Pharmacopoeia, 2019) . The intermediate precision for three replicates each day and each analyst were evaluated using standard deviation (SD) (Chitsopa K, 2019) . Acceptable SD was defined as being 0.3 or smaller (Chitsopa K, 2019) . The results showed the SD values of the three different days by the main analyst as 0.09, 0.08 and 0. 11, respectively. The SD values for the other two analysts were 0. 10 and 0. 03 respectively. Due to the very low water content of erythropoietin products and inconsistency of water amount in different individual vials, we considered it acceptable to use % margin as acceptance criteria for intermediate precision testing. The results revealed the excellent % margin of 6% which was extremely lower than the acceptance criteria of 25% (Little TA, 2016).

Table 3. SD and % margin of the intermediate precision

Day	**Average water content [%]**	**SD**
1	0.5	0.09
2	0.7	0.08
3	0.5	0.11
Analyst 2	0.7	0.10
Analyst 3	0.5	0.03
Average water content [%]	0.6	
SD of water content	0.08	
Factor for SD	2.575	
Limit [%]	4	
% Margin	6	

Conclusion

The analytical method for water determination in the lyophilized erythropoietin products using external extraction and volumetric KF titration technique was developed and validated. Hydranal™ formamide dry and the mixture of Hydranal™ methanol dry and Hydranal™ formamide dry in the ratio of 60:40, were selected to be used as the external extraction solvent and the solvent in the titration vessel, respectively. The validation data shows that the method was specific, precise, accurate, and reproducible. Hence, the developed and validated method in this study is suitable for determination of the residual water content in the erythropoietin products in order to ensure the products' quality, efficacy and stability in routine analysis.

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