Optimization Sonication Time and Dilution Factor in Determining the Concentration of Endotoxin Challenge Vial with Kinetic Turbidimetric Method

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Abstract. ECV has a specification certificate of analysis (CoA) worth 9616 EU/mL where the limit of acceptability recovery is in the range of 50 – 200 percent (4808 EU/mL - 19232 EU/mL). The verification results of ECV concentration with kinetic turbidimetry method showed recovery outside the lower limit of the predetermined range less than 50 percent. This is due to endotoxin adsorbed onto the surface of the glass. To get a recovery that goes within the limits of acceptability then do the time variation of sonication (5; 10; 15; 20; 25; 30) minutes and variations of the dilution factor of $10^{-4}$; $10^{-5}$. The result showed that the optimum sonication time was 25 minutes with a $10^{-4}$ dilution factor. The average concentration of ECV obtained worth 7828.98 EU/mL with a recovery 81.4 percent. The validation test showed a significant correlation coefficient values (-0.9963); and the precision of data with CV < 2/3 CVH, control of graph showed that the data was homogenic; the value of LOD (1070.6823 EU/mL); LOQ (3568.9411 EU/mL) and percent recovery value was 81.0844 percent. Suggested for further research to optimize the use of buffer solution as a solvent, but it also can optimize the temperature and the frequency of the sonication process.

1. Introduction
Depyrogenation is an important factor in maintaining guarantee of sterility during the preparation of parental pharmaceutical products. Sterility indicates whether a sterile material or product is free from microorganism contamination. However, at one stage of the production process such as sterilization, parental products are sometimes contaminated by endotoxins. Giving drugs that are contaminated with endotoxins can cause pyrogenic reactions, complications and even death to patients. Depyrogenation can be defined as the elimination of all pyrogenic substances, including bacterial endotoxins, and is generally achieved by the removal or inactivation of pyrogenic substances [1]. In depyrogenation studies used endotoxin indicators which are an important component to show the performance of depyrogenation devices.

The endotoxin indicator is made from the Endotoxin Control Standard (SKE) of the Escherichia coli lipopolysaccharide. Besides SKE, there is also an Endotoxin Challenge Vial (ECV). One of the
producers produces ECV with an average concentration specification for each vial of 9616 EU / vials (based on Certificate of Analysis). This value needs to be verified because the producer is testing with the Chromogenic Kinetic method while the user laboratory uses the Turbidimetric Kinetic method. After the verification test, recovery (recovery) showed that the concentration of ECV that did not meet the CoA specifications was 4576 EU / mL (47.59%). Meanwhile, according to USP <3299>; <85> recovery of endotoxin concentrations must be in factor two of the labeled endotoxin concentrations or in the range of 50% - 200% (4808 EU / mL - 19232 EU / mL). Recovery of less than 50% was caused by endotoxins which were adsorbed onto the glass surface and not all of them can be recovered [2]. Ultrasonic method in order to obtain optimum recovery endotoxin [3]. According to [4] sonication can accelerate the extraction of endotoxins from ECV. However, sonication was also a method for sterilization. Therefore, the research was conducted to find the optimum sonication time and dilution factor so that recovery falls within the CoA specification range.

2. Material and Method
This type of research is an experiment, which is a study by conducting experimental activities aimed at finding certain treatments for others in controlled conditions. In this study Pretest-Posttest Control Group Design is used which compares the initial test results (before treatment is given) with the final test results (after treatment is given). The analytical method in this research is the Turbidimetry Kinetic using a Toxin meter measuring tool that follows the photometry principle based on the speed of time of gel formation. The toxin meter will tell when the gel is formed (T-Gel). The higher the endotoxin concentration, the lower the reaction time and vice versa. The toxin meter measures the transmittance ratio of the well independently and simultaneously. The reaction time is determined when the transmittance ratio decreases in value to the threshold [5].

Endotoxin control standards (ECS) were used as ingredients to make calibration curves and pyrogen-free water as solvents and thinners. The sample population in this study was Endotoxin Challenge Vial (ECV) with Endotoxin samples and Limulus Amoebocyte Lysate (LAL) reagents. LAL can detect and analyse quantitative bacterial endotoxins. LAL utilizes the basic immune response of horse landed crabs against invasion of Gram-negative bacteria [6]. The ingredients contained in amoebocytes of horse landed crabs consist of various proteins, factors, cofactors and ions that interact to cause coagulation. Gram-negative endotoxins catalyse the activation of proenzymes in the Limulus amoebocyte lysate. The initial speed of activation determined by endotoxin concentration [7]. Furthermore, activated enzymes (coagulase enzymes) hydrolyse specific bonds in a coagulating protein (coagulate) which is also present in the limulus amoebocyte lysate to produce coagulin [8]. Once hydrolysed, the resulting coagulin combines by itself and forms a lump or clot like gel. This research was started by making a calibration curve, then preparing test samples with certain treatments followed by measuring the time of gel formation on the toxin meter so that the concentration of the test sample can be calculated. Endotoxin concentrations are calculated based on extrapolating the linear equation from the logarithmic curve using the formula:

\[
\log T_{Gel} = b \log C + a
\]

\[
\log C = \frac{\log T_{Gel} - a}{b}
\]

\[C = \text{Anti Log (Log C)}\]

\[T_{Gel} = \text{time of gel formation}\]
\[a = \text{Intercept kurva}\]
\[b = \text{Slope kurva}\]
\[C = \text{Endotoxin concentration}\]
Endotoxin concentrations were expressed as Endotoxin Units per mL (EU / mL). One unit of endotoxin / mL (EU / mL) was equivalent to 0.1 ng endotoxin / mL of solution. Where 1ng / mL was equivalent to 1 ppb or $10^{-9}$ so that 1 EU / mL was equivalent to $10^{-8}$. The calibration curve was made from the retail series ECS with a concentration of 5; 0.5; 0.05; 0.005 EU / mL with respect to the time of gel formation (T-Gel), then a linear regression equation was made. Endotoxin Control Standards (ECS) calibration curve was said to be linear if the correlation coefficient ($r$) $\leq -0.980$. To get the optimum sonication time and dilution factor, 7 vials of Endotoxin Challenge Vial (ECV) were provided; each added 1 mL of pyrogen-free water; vortex for 30 minutes. One vial Endotoxin Challenge Vial (ECV) was prepared as a comparison (without sonication) while the remainder were each sonicated for 5; 10; 15; 20; 25 and 30 minutes. After sonication process was complete, each ECV solution was diluted using pyrogen-free water with dilution factors ($f_p$) $10^{-4}$ and $10^{-5}$.

The measurement of the formation time of the test sample gel was duplicated by piping each 100 µL of the test sample and then adding 100 µL of LAL reagent to all tubes, carefully homogenized using a vortex mixer for 2-3 seconds. Then put into the Toxin meter analysis module and incubated for 60 minutes at 37 ± 0.5°C. After the measurement was finished, it was read and recorded the gel formation time of each solution. Determination of endotoxin concentration was calculated according to the dilution factor. Based on the concentrations obtained, a control chart is made to determine the optimum sonication time and dilution factor so that validation methods can be performed including Linearity; Range; Sensitivity; Accuracy; Precision; LOD and LOQ.

3. Result and Discussion

3.1 The Logarithm calibration curve of ECV concentration against T-Gel formation

The calibration curve shows linearity, range and sensitivity where a regression equation was obtained, $Y = -0.2588X + 0.9895$ with a correlation coefficient ($r$) -0.9963 and a sensitivity of 0.2588. The negative correlation coefficient shows that the two variables have a reversal relationship. This means that the longer the formation time of the gel, indicated the lower of ECV concentration.

3.2 Optimization results of sonication time and dilution factors.

Determination of the optimum sonication time and dilution factor by making a control chart in order to obtain the maximum concentration of ECV that meets the CoA specifications (4808 - 19232 EU / mL). The optimum sonication time and dilution factor reach in 25 minutes with $10^{-4}$ dilution factor. Graphs of ECV analysis based on time variations and dilution factors are available in Figure 1.

![Figure 1. Optimization of sonication time and dilution factors](image-url)
The figure showed the optimum time of sonication at the 25 minutes point with a dilution factor of $10^{-4}$ so that a maximum concentration of 7828.9786 EU / mL was obtained with recovery of 81.42%. The treatment without sonication at dilution $10^{-4}$ gave ECV concentrations of 4503.66 EU / mL, while at dilution $10^{-5}$ gave lower ECV concentrations of 2997.37 EU / mL. The acquisition of ECV without sonication has not given results in accordance with the CoA specifications (4808 - 19232 EU / mL). ECV concentrations with a $10^{-4}$ dilution from sonication time of 5 minutes to 30 minutes give results that are in accordance with CoA specifications, with an optimum sonication time in 25 minutes. Whereas in diluting $10^{-5}$ results of sonication from 5 minutes to 30 minutes, there were no sonication time that gave ECV concentrations according to the CoA specifications.

3.3 Validation of the method
It was known that dilution $10^{-4}$ gives optimum results at sonication time of 25 minutes for ECV analysis, therefore the method will be validated in order to provide scientific study results. The validation parameters of the analysed method were linearity and range, accuracy, precision, control diagram, LOD and LOQ. Samples used for the validation of the method were 7 samples which were treated with 25 minutes sonication time with $10^{-4}$ dilutions. Method validation shows an accuracy test with 81.0844% recovery, so it can be stated that the test results are accurate and meet the CoA specifications; precision test obtained% RSD (4.5773%) < $\frac{2}{3}$ CVH (5.5369) then the data is precise; have a LOD value (1070.6823 EU / mL); LOQ (3568.9411 EU / mL); the data obtained is in the range of 3SD and -3SD, the data was homogeneous. Data of validation method showed in Table 1.

| Table 1. Measurement of ECV (EU/mL) for validation method |
|-----------------|-----------------|-----------------|-----------------|
| Validation parameter | Sample | Sample + Spike |
| | T-Gel ($N=7$) (minutes) | ECV ($N=7$) EU/mL | T-Gel ($N=7$) (minutes) | ECV ($N=7$) EU/mL |
| Average | 10.4±0.1 | 7797.08±356.9 | 8.8±0.1 | 14950.01±729.8 |
| %RSD | 4.57% | |
| 2/3 CVH | 5.54% | |
| LOD | | 1070.68 EU/mL |
| LOQ | | 3568.94 EU/mL |
| % Recovery | | 143.06% |
| Specification CoA (EU/mL) | | 9616 EU/mL |
| % Recovery CoA (50-200) % | | 81.08% |

Basically, this study aims to verify the concentration of Endotoxin Challenge Vial (ECV). Verification test is very important to be done on new products or test materials that have CoA. Because before the product is released, specifications must be ensured that it can be used for routine testing. The difference in the method of analysis of ECV producers with users causes recovery not meeting CoA specifications. This is biased because each analysis method has a different sensitivity depending on the reagent material (reagent) used. Accuracy test was obtained from the test for interfering factors by spiking the standard control of endotoxin concentration in samples with a concentration of 5 EU / mL. This is done to analyse the efficiency of recovering added analytes so that their accuracy can be calculated.

ECV producers do the testing with the Chromogenic Kinetic method while the user laboratories use the Turbidimetric Kinetic method. Reagents for the Chromogenic Kinetic method contain synthetic chromogenic peptides as substrates against clotting enzymes in a coagulate protein so that the chromogenic substrate is hydrolysed and releases the final yellow chromogenic group [9]. Whereas the reagent for the Turbidimetry Kinetic method does not contain a substrate with the principle of absorbing and scattering light during a gel reaction to produce a signal [8].
Verification test results, recovery (recovery) shows the concentration of ECV that does not meet the CoA specifications of 4576 EU/mL (47.59%). Meanwhile, according to USP <3299>; <85> recovery of endotoxin concentrations must be in factor two of the labelled endotoxin concentrations or in the range of 50% - 200% (4808 EU/mL - 19232 EU/mL). Recovery of less than 50% is caused by endotoxins which are adsorbed onto the glass surface and not all of them can be recovered. This is caused by the lipid compound A from hydrophobic endotoxins which makes it difficult to dissolve. It was also caused by the attractive attraction between endotoxins and the glass surface where they will find balance [9].

The sonication extraction method utilizes ultrasonic waves with a frequency of 42 - 48 kHz which can accelerate the contact time between the test sample and the solvent even at room temperature. This causes the process of mass transfer of bioactive compounds from endotoxins to solvents to be faster. Sonication relies on the wave energy that causes the cavitation process, which is the process of forming small bubbles due to ultrasonic wave transmission to help the diffusion of solvents to the sample to be extracted [10].

In this study there are things that can affect the validity of the test results. Therefore, it is very important to note, among others [11]:

- The use of PPE such as masks and gloves were very important to avoid exogenous endotoxins that can contaminate test samples and reagents.
- Measurable devices such as micropipettes, toxin meters and ultrasonic devices must be calibrated to ensure the traceability of each device.
- The toxin meter must be stable at 37 ± 0.5°C
- Pay attention to batch numbers and SKE expiration dates, LAL reagents and pyrogen free water.
- Amoebocyte lysate (LAL) limousine and endotoxin control standard (SKE) must be stable.
- When plotting to dilute the SKE solution and the test sample must use a different Finn tip for each retail series.
- Standard endotoxin control calibration curves with:
  - Slope (b) = -0.1 <b <0.1 and correlation coefficient (r) ≤ -0.980.
  - Gel formation time (T gel):
  - The T control gel was negative > 60 minutes, whereas the T Gel test sample was within the standard curve range.

In this study there are shortcomings where researchers cannot ensure that ultrasound was calibrated. This causes researchers to not know the achievement of traceability and determine the deviation (deviation) of a measurement. Because keep in mind that each instrument, including ultrasound, has a frequency in the use of a certain period can decrease its stability. It is recommended that further researchers be able to ensure that the ultrasound was calibrated.

4. Conclusion
Based on the results of research and data processing in determining the ECV concentration of the turbidimetric kinetic method it can be concluded that the optimum sonication time is 25 minutes with a dilution factor of 10 giving ECV concentrations in accordance with CoA specifications with a% recovery of 81.42%. In accordance with the results of the method validation, the data gives precise and accurate results and LOD and LOQ values that are still readable by turbidimetry.

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