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Validation of moist and dry heat processes used for sterilization and depyrogenation during ampoules manufacturing

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Abstract

Pyrogens are fever-producing substances, which are metabolic products of microorganisms. Endotoxins are the most potent pyrogens. Depyrogenation can be defined as the elimination of all pyrogenic substances which is an important part of the manufacture of sterile pyrogen free pharmaceutical products. This study compared between sterilization and depyrogenation using moist heat by autoclave, as well as dry heat. After incubation period (24 hours) at 55 ^{o}C-60 ^{o}C the color of *Geobacillus stearothermophillus* spores' control vial which didn't sterilized in autoclave changed from blue to yellow color representing the (+ve) result, while the other sterilized vials have no color change (-ve). In addition, all examined endotoxin containing ampoules showed gel formation (+ve) when examined by Limulus Amebocyte Lysate (LAL). All ampoule groups that have autoclaved for periods of 1 hour to 4 hours showed gel formation (+ve) after LAL test, while only the last group which have autoclaving periods of 5 hours (5 cycles) showed no gel formation (-ve). Sterility of vials that contained spores of *Geobacillus stearothermophillus* after dry heating at 250 ^{o}C for 30 min showed no color change (-ve). In addition, LAL test for endotoxin containing ampoules after dry heating at 250 ^{o}C for 30 min showed no gel formation representing (-ve) pyrogen. So, moist heat is effective in sterilization and not for depydrogenation, whereas, dry heat is effective in sterilization as well as for depyrogenation.

Key words
Sterilization, Dry and Moist heat, Depyrogenation, Ampoule, Endotoxin

1. Introduction

The most effective way to destroy microorganisms is through "heat", as it coagulates their proteins as well the enzymes present in them. So sterilization (destroying or killing the microorganism) process follows this principle of killing microorganisms, which can be either by giving wet (moist) heat or dry heat [1].

Heat sterilization methods using moist heat or dry heat sterilization technique are the effective methods used to sterilize pharmaceutical products which are heat stable (thermostable) products while thermolabile products sterilized with other methods like radiation sterilization or chemical gases sterilization [2]. Thermolabile pharmaceutical products are these products, which require special storage conditions (cold storage) at specific temperatures below room temperature. Moist heat sterilization methods are important for sterilizing instruments, tools and pharmaceutical products. The autoclave with steam pressure and relatively high temperature lower than that of dry heat sterilization is used in moist heat sterilization [1]. Moist heat sterilization mechanism in sterilizing the equipment and pharmaceutical products is the denaturation of the microorganism's proteins structure and the enzymes of microorganisms present on the equipment or pharmaceutical product and thus killing them. Moreover, the required time for moist heat sterilization is about 15-20 minutes with the temperature of 121 °C [3].

Dry heat sterilization is one of the oldest techniques used to sterilize the glassware and other equipment. In this method, dry heated air of high temperature is used. Heat is moved through air from the surrounding area of the equipment and transferred to the next layer, whereas, slowly the whole equipment gets heated and sterilization is achieved. The sterilization time may be last from 1 to 2 hours period with the temperature of the 160 °C to 170 °C sequentially [4]. Dry heat sterilization temperature is higher than that of moist heat sterilization process; there are more chances for destroying the microorganisms.

Pyrogens are fever-inducing substances, which considered as a metabolic biproducts of microorganisms. Chemically, they are lipid in nature and associated with a polysaccharide carrier molecules [5].These polysaccharide carriers enhance the lipid's solubility. Microorganisms including bacteria, yeasts and moulds are producing pyrogens in surrounding media. Endotoxins are the most potent pyrogens, have a high molecular weight and produced from the cell walls of Gram-negative bacteria [6].

There are two phases of endotoxins' presence in environment it may be associated with live organisms or in a free form (unassociated). Endotoxin associated with microorganisms may be removed by bacterial filtration using microporous sterilizing filters. However, the other free forms of endotoxin can't be

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retained by bacterial filtration because it passes through these filters' pores. In addition, it cannot be destroyed by ordinary moist heat autoclaving cycle (121 °C for 15-20 min) as it is heat stable. Endotoxin can be present in food, drinking waters, soil and substances. It is also present on laboratory glassware, solid parts, apparatus, ampoules, tools and water baths. Endotoxin affects human body by inducing high fever, diarrhea, low blood pressure, and fetal shock syndrome. Due to high risk of contamination, pharmaceutical water systems, medical devices and pharmaceutical parenteral products are tested for presence or absence of endotoxins [7]. The high variability of pyrogen's molecular weight causes it very difficult to be removed from solutions. Endotoxins are relatively heat resistant and insensitive to pH changes, however, several removal techniques exist [8, 9]

Endotoxins present in all moisture places [10]. Endotoxin reduction is assessed using endotoxin indicators, Control Standard Endotoxin (CSE) derived from *Escherichia coli* O113:H10 used to prepare these indicators. The prepared CSE should have the same effect of endotoxin used to perform routine the Bacterial Endotoxin Test (BET) using Limulus Amoebocyte Lysate (LAL) methodology and traceable to a reference standard. Rabbit pyrogen test and Limulus Amoebocyte Lysate (LAL) assay are the most used techniques for endotoxin detection and approved from FDA [10]. Nowadays, tests of endotoxin detection that based on LAL are the most popular. LAL is derived from the blood of horseshoe crab, *Limulus polyphemus*, and clots when exposed to endotoxin [11]. LAL assay test is the LAL gel-clot formation. So that, when LAL reagent is mixed with a sample containing endotoxin, a gel will be formed. The absence of endotoxin is a negative reaction (no clot is obtained) [11].

Depyrogenation of pharmaceuticals is very important to avoid pyrogenic effect of endotoxin during the manufacturing of pharmaceutical products and is distinct from sterilization. Endotoxin is one toxin that is highly heat stable and not destroyed by standard sterilization cycles (e.g., autoclaving). If only sterilization is required to be validated, this can be examined using biological indicators impregnated with endospores from a heat resistant bacteria; e.g., *Bacillus subtilis* var. *niger* [usually used for dry heat] or *Geobacillus stearothermophilus* [usually used for moist heat, although the microorganism also has a high resistance to dry heat] [12]. Depyrogenation can be defined as the removal or inactivation of all pyrogenic substances, including bacterial endotoxin [13, 14]. Destruction of endotoxin by moist heat is almost very difficult to be realized, and has low log reductions when related to that of dry heat [15]. Dry heat depyrogenation for glass and stainless steel tools in the pharmaceutical manufacturing industry is the major endotoxin destruction technique used [16]. This process sterilizes and depyrogenates and is mainly used for glass ampoules and other tools.

Productions of glass ampoules are normally in two types; one is empty closed ampoules and the other one is empty opened ampoules. Opened empty ampoules should be washed and depyrogenated before entrance to filling area due to risk of microbial contamination during handling and transferring, while empty closed ampoules need only to depyrogenated before filling, as there is no risk of inner microbial contamination. Bothe closed and opened empty glass ampoules are placed in the dry heat-annealing tunnel at approximately 600 °C during production before transferring to pharmaceutical manufacturer site. The aim of this study is to compare between the effects of moist and dry heat on depyrogenation of endotoxins inside glass ampoule, as well as, validate ampoule of not being have any pyrogen.

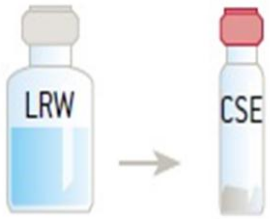
2. Materials and methods

1. Preparation of test ampoules containing *E. coli* Control Standard Endotoxin (CSE)

1. We get Control Standard Endotoxin vial (CSE) LOT. NO. 149 which produced by Associates of Cape Cod, Inc. through its agent in EGYPT Advanced Technology Group.
2. Reconstitute the vial of endotoxin (LOT. NO. 149) with 2.5 ml LAL Reagent Water (Plate Ia).
3. Vortex the vial of endotoxin for at least 15 minutes.
4. The vial will be 5000 EU/2.5 ml =1000 EU/0.5 ml.
5. Put 0.5 ml from the reconstituted (CSE) in each empty opened ampoule, covered with aluminum foil, and dried in dry heat oven at 60^{o}C for complete drying (2–3 Hr.) actual 2:40 hr.
6. Close ampoules before going to depyrogenation test, by the manufacture.

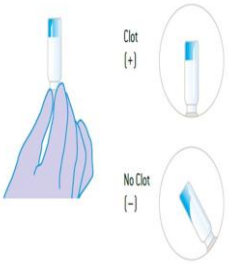
2. Limulus Amebocyte Lysate (LAL reagent) for testing endotoxin

1. We get Lyophilized Lysate Reagent LOT. NO. S15-802, which is compatible with endotoxin LOT. NO. 149 and produced by Associates of Cape Cod, Inc. through its agent in EGYPT Advanced Technology Group (LAL vial) (Plate Ib).
2. Reconstitute the ampoule to be tested (containing endotoxin) with 1 ml LAL Reagent Water (LRW) and vortex for 1 min.
3. Transfer 0.25 ml of (LRW) from the tested ampoule containing endotoxin after reconstitution to be tested in the vial of LAL reagent.
4. Mix by tilting and gently swirl the vial until the contents are in solution (Plate Ib)
5. Immediately incubate each vial of LAL at $37^{o}C\pm1^{o}C$.
6. After 60 min (± 2 min.) of incubation, carefully remove each vial and invert 180^{o} , record the reaction in each vial as either positive for gel formation (clot) or negative (no clot)(Plate Ic).



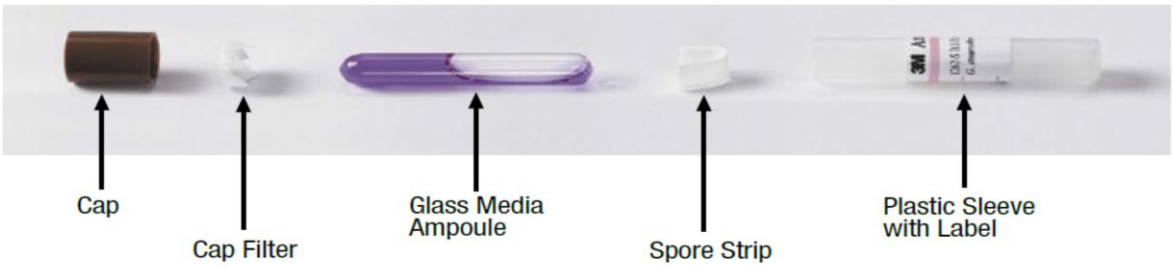
(a)Vial of endotoxin

(b) Mix by tilting and gently swirl the vial of LAL

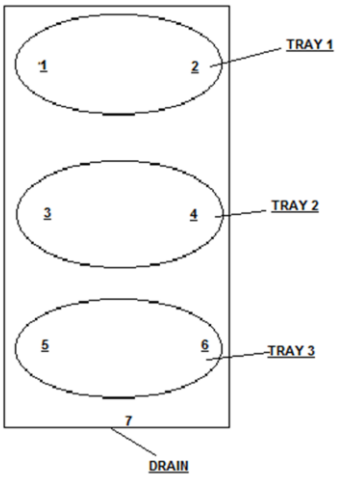


(c) Positive for endotoxin as gel formation (clot) or negative (no clot)

7953 spores, a glass ampoule containing recovery medium which includes a pH indicator dye, a plastic cap with filter,



(d) Biological indicator EZ Test into a thermoplastic vial manufactured by Mesa Laboratories



(e) Distribution of the biological indicator and the endotoxin ampoules in autoclave chamber

(f) Ampoules entrance to oven

Plate I. Sterilization and depyrogenation tests

3. Comparison between sterilization and depyrogenation using moist heat (autoclave)

1. In the experiment we used EZ Test contains bacterial spores (*Geobacillus stearothermophillus* lot No.: S-495 with heat shocked population 2 X 10^6 cfu/stripe) on a filter-paper carrier packaged within a small, thermoplastic vial culture tube manufactured by Mesa Laboratories [17] (Plate Id). Inside the thermoplastic vial culture tube is a sealed-glass ampoule of specially formulated soybean casein digest culture medium containing a color indicator (blue color), which turns a dramatic yellow (in normal growth of spores) when spores mixed with medium and grow. EZ Test is easy-to-use and produces visual results within 24 hours.
2. Distribute 7 vials of *Geobacillus stearothermophillus* in autoclave where two in each tray and one at the bottom of the chamber near the drain point (Plate Ie).
3. Distribute 7 endotoxin ampoules (1000 EU as prepared above) in autoclave two in each tray and one at the bottom of the chamber near the drain point (Plate Ie).
4. Operate the autoclave on sterilization cycle (121°C for 20 min.)
5. After sterilization cycle end remove the 7 *Geobacillus stearothermophillus* spores' plastic vials from the autoclave and allow cooling for at least 10 min, in addition, remove the 7 endotoxin ampoules.
6. Observe the color change of the *Geobacillus stearothermophillus* spores' vials indicator from blue (color before entrance oven) to green/gray color due to exposure to steam.
   Extended exposure will result in further change to a black color. The purpose of chemical indicator is to distinguish exposed from unexposed unites.
7. Gently squeeze the crusher to break the glass ampoule of each plastic vial, this will allow the growth media to meet the spores strip of the microbe (*Geobacillus stearothermophillus* spores).
8. Incubates the *Geobacillus stearothermophillus* spores' vials at 55-60^{o}C for 24 hours.
9. Incubate a control *Geobacillus stearothermophillus* spores' vial (without sterilization) to compare color change (yellow color for control of normal spores growth).
10. After removal of the 7 endotoxin ampoules, do the LAL test as mentioned above.

4. Depyrogenation by using moist heat (autoclave) at different periods of autoclaving (More than one cycle of autoclaving)

1. Put group No. 1 (7 ampoules) in autoclave two ampoules in each tray and one at the bottom of the chamber near the drain point the same as in Plate Ie.
2. Operate the autoclave on sterilization cycle at 121^{o}C for 1hr (cycle No. 1).
3. After the end of cycle No.1 remove the endotoxin ampoule and do LAL test as mentioned before.
4. The other ampoule groups of 2, 3, 4 and 5 numbers the same procedures of sterilization in autoclave were done with different autoclaving periods 2, 3, 4, 5 hours, respectively; in addition, LAL test was performed for each group.

5. Sterilization and depyrogenation using dry heat (oven) at (250 ^{o}C for 30 min)

1. Put 5 vials of *Geobacillus stearothermophillus* (as previously mentioned) and 5 endotoxin ampoules (1000 EU as prepared above) on the oven's belt at different 5 distance of locations (Plate If).
2. Operate the oven at (250 ^{o}C for 30 min)
3. After ampoules and vials exit from the oven, remove the 5 *Geobacillus stearothermophillus* spores' vials from the belt and allow cooling for at least 10 min, in addition transfere the 5 endotoxin ampoules to be ready for LAL test.
4. Observe the color change of the *Geobacillus stearothermophillus* spores' vials from blue (color before entrance oven) to green/gray color due to exposure to heat.
5. As manufacture guidelines, gently squeeze the crusher to break the glass ampoule. This will allow the growth media to encounter the spores strip. Incubates the *Geobacillus stearothermophillus* spores' vials at 55-60^{o}C for 24 hours. *Geobacillus stearothermophillus* spores' vial without sterilization was used as control to compare the change in color.
6. The removed 5 endotoxin ampoules were tested for pyrogen by LAL test as previously mentioned.

3. Results

3.1. Comparison between sterilization and depyrogenation using moist heat (autoclave)

After incubation period (24 hours) for the biological indicator at 55 ^{o}C - 60 ^{o}C the color of *Geobacillus stearothermophillus* spores' control vial which didn't sterilized in autoclave changed from blue to yellow color which mean (+ve) result, while the other 7 *Geobacillus stearothermophillus* spores' vials which sterilized have no color change (-ve) result **(Table 1)**. On the other hand, all examined endotoxin-containing ampoules showed gel formation (+ve) as in **(Table 1)** meaning the pyrogen still present.

3.2. Depyrogenation using moist heat (autoclave) at different periods of autoclaving (Cycles)

As shown in **(Table 2)**, all ampoule groups No. 1,2,3,4 that have autoclaved for periods of 1 hour to 4 hours showed gel formation (+ ve) after the test of LAL. On the other hand, only group No. 5 which have autoclaving periods of 5 hours (5 cycles) showed no gel formation (-ve) and the depyrogenation occurred **(Table 2)**.

3.3. Test of sterilization and depyrogenation using dry heat (oven) at 250°C for 30 min

After incubation period of 24 hours at 55-60 °C the bacterium spores of *Geobacillus stearothermophillus* control vial which didn't sterilized in dry heat oven, the color of this control vial change from blue to yellow which mean (+ve) result, while the other 5 vials of *Geobacillus stearothermophillus* spores' have no color change showing negative results (-ve) **(Table 3)**.

Table 1. Effect of moist heat by autoclaving at 121°C for 20 min on sterilization of *Geobacillus stearothermophillus* spores' vials and depyrogenation of endotoxin ampoules (LAL testes).

Location No.	*Geobacillus stearothermophillus spores' vials test*	*LAL test*
1	- ve	+ ve
2	- ve	+ ve
3	- ve	+ ve
4	- ve	+ ve
5	- ve	+ ve
6	- ve	+ ve
7	- ve	+ ve

+ve: Positive for gel formation (clot) -ve: negative for gel formation (no clot)
+ve: Positive for growth (color change) -ve: negative for growth (no color change)

Table 2. Effect of moist heat by autoclaving at 121°C on depyrogenation of endotoxin in ampoules at different autoclaving periods.

Ampoule number	*LAL test for ampoule groups*				
	No. of group /Autoclaving periods				
	Gp.1/1 hr.	*Gp.2/2 hr.*	*Gp.3/3 hr.*	*Gp.4/4 hr.*	*Gp.5/5 hr.*
1	+ ve	+ ve	+ ve	+ ve	-ve
2	+ ve	+ ve	+ ve	+ ve	- ve
3	+ ve	+ ve	+ ve	+ ve	- ve
4	+ ve	+ ve	+ ve	+ ve	- ve
5	+ ve	+ ve	+ ve	+ ve	- ve
6	+ ve	+ ve	+ ve	+ ve	- ve
7	+ ve	+ ve	+ ve	+ ve	- ve

+ve: Positive for gel formation (clot) -ve: negative for gel formation (no clot)

Table 3: Effect of dry heat oven at 250 °C for 30 min on sterilization of *Geobacillus stearothermophillus* spores' vials and depyrogenation of endotoxin ampoules (LAL testes).

Location No.	*Geobacillus stearothermophillus spores' vials test*	*LAL test*
1	- ve	- ve
2	- ve	- ve
3	- ve	- ve
4	- ve	- ve
5	- ve	- ve

+ve: Positive for gel formation (clot) -ve: negative for gel formation (no clot)
+ve: Positive for growth (color change) -ve: negative for growth (no color change)

In addition all endotoxin containing ampoules show no gel formation (-ve) when LAL test was examined **(Table 3)** representing no pyrogen.

4. Discussion

Sterilization refers to the destruction of living microorganisms and not necessarily destroying its microbiological bi-products or toxins. The production of sterile pharmaceutical dosage forms is considered as a big issue have a high risk of microbial contamination which surely affect the patient health, The presence of endotoxin/pyrogens in sterile pharmaceutical products offerings a major risk to patients more than microbial contamination risk. The main source of endotoxin in pharmaceutical products is pharmaceutical grade water, due to the possibility presence of Gram-negative bacteria, which acts as the natural bacterial source of endotoxins after rupture of its cell wall. The water source should be treated to produce pyrogen free Water-For-Injections (WFI), because it is the major ingredient used in manufacturing of sterile pharmaceutical solutions. The best method to discover endotoxin presence in water is the Limulus Amebocyte Lysate (LAL) test [18].

Depyrogenation (removal of endotoxin) is a great subject to be argued and numerous methods used to remove it to obtain pyrogen free product, these methods vary between using chemicals, radiations, ultrafiltration and heat treatment. Dry heat depyrogenation is the maximum current method used in pharmaceutical manufacturing mainly with solid parts/tools. Moist heat is greatly more effective as a sterilization method not as depyrogenation method which is consistent with the result of this study [19].

Sterilization techniques are wide-ranging between physical sterilization (heat and radiation), chemical sterilization (formalin and ethylene oxide) and mechanical sterilization (bacterial filters). These techniques can kill or remove and retain microorganisms whereas, endotoxins/pyrogens may fight these methods, so when we observe the difference between sterilization and depyrogenation using autoclave we found that moist heat sterilization at autoclave (121°C for 20 min) is enough for sterilization and not enough for depyrogenation [20]. Result of this study is considered as practical evidence to prove moist heat sterilization concept, which used in many pharmaceutical companies.

Moist heat sterilization (autoclaving) at 115–118 °C for 30 min or 121–123 °C for 15 min or an appropriate temperature/time cycle is effective in killing microorganisms [21], so in this study when we expose ampoules containing endotoxin for moist heat sterilization by autoclave at (121°C) we need 5 hours for depyrogenation and destruction of endotoxin. The same was reported previously, where, prolonged periods of moist heat autoclaving or at high temperature exposure more than 121 °C may be enhanced depyrogenation [9, 12]. Moreover, endotoxin might be destroyed by moist heat, only with very low concentration of endotoxin and is used with heat non-stable materials. Destruction of endotoxin by moist heat is almost very difficult to be realized, and has low log reductions when related to that of dry heat [15].

Practically moist heat depyrogenation at (121 °C) for 5 hours is not effective when compared with dry heat sterilization at 250 °C or above [22, 23], due to presence of many disadvantages as follow:

1. Economically very expensive to use autoclave for long period cycle to carry out depyrogenation
2. Affecting pharmaceutical product -if present – which can't resist temperature for these long time (5 hours)
3. Time consuming
4. Need more labors
5. Power consuming
6. Decrease working life of autoclave
7. Maintenance cost
8. Low production capacity

Therefore, moist heat is effective in sterilization especially for terminally sterilized pharmaceutical products and not for depyrogenation [24], whereas, dry heat is effective in sterilization and depyrogenation. Items which sterilized by dry heat may be glass containers, stainless steel equipment, and/or dry powders, if the powder is thermostable (can resist exposure to high-temperature). Practically we cannot use moist or dry heat in sterilization of thermolabile pharmaceutical product in its final container (Aseptic filled product) [25].

Results of this study reported that success depyrogenation occurred for endotoxin containing ampoules after dry heating at 250 ^{o}C for 30 min. Dry heat involves exposing the parts to a high level of heat (generally in-between 180 °C and 250 °C) for a specific time (the higher temperature used, the smaller period needed). The usual used cycle is 250 °C for not less than 30 minutes as we examined in our study. For example, the European Pharmacopoeia in chapter 2.6.8 recommends two possible time-temperature combinations for endotoxin destruction: 60 minutes at 200 °C or 30 minutes at 250 °C. The amount of endotoxin destroyed at 250 °C for 60 minutes would not essentially wholly destroyed at 200 °C for 60 minutes, based on the non-linearity of the heat destruction curve, whereas, endotoxin destruction at low temperature is of the second choice [26].

Actually, dry heat is reflected the greatest effective technique in depyrogenation (destroying endotoxins) while temperatures required confirming the depyrogenation process for glass containers ampoules are a minimum of 250 °C. Therefore, dry heat depyrogenation needs higher temperatures and longer exposure times than that necessary for dry heat sterilization. One of dry heat sterilization advantages is that materials/tools outcome dry after end of the cycle, so no decomposition of steel materials. On the other hand, the disadvantages of dry heat sterilization is that the process is difficulty controlled due to particular temperature limits [19].

So, when we compare our results with the recently and traditional published articles, we can prove that the most preferable method for sterilization of thermostable pharmaceutical products or tools is moist heat while dry heat is the best choice for depyrogenation [27].

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