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Depyrogenetion Methods

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Abstract

Pyrogens are fever-and inflammation-causing agents that cause serious health hazard, especially in the case of intravenous drugs and pharmaceutical products. While lipopolysaccharide (LPS) from the outer membrane of Gram-negative bacteria remains the prototypic pyrogen, substances such as lipoteichoic acid (LTA) and peptidoglycan from Gram-positive bacteria and others are increasingly recognized as potent immune stimuli. The presence of pyrogens in the body initiates secretion of pro- inflammatory cytokines by monocytes. When high concentrations of pyrogens enter the blood stream they cause fever, septic shock or even death.

The presence of small amounts of endotoxin in recombinant proteins preparations can cause side effects in host organism such as endotoxin shock, tissue injury, and even death. Due to these reactions, it is essential to remove endotoxin from drugs, injectables, and other biological and pharmaceutical products. Depyrogenation refers to the removal of pyrogens from solutions, most commonly from injectable pharmaceuticals.

A depyrogenation study is the key biological test, in addition to thermometric tests, for the qualification of depyrogenation devices. Depyrogenation can be defined as the elimination of all pyrogenic substances, including bacterial endotoxin, and is generally achieved by removal or inactivation. Depyrogenation, like sterilization, is an absolute term that can only be theoretically demonstrated because of test insensitivity. Because virtually all new materials involved in a production process, including factory employees, can be potential sources of pyrogen contamination, raw material screening and depyrogenation can often go a long way to ensure that final products are free from pyrogens and does not require costly removal of or inactivation of pyrogen. The current review provides a detailed discussion of the theoretical concept and mechanism of depyrogenation.

Introduction

Pyrogenicity

Pyrogens are fever-and inflammation-causing agents that cause serious health hazard, especially in the case of intravenous drugs and pharmaceutical products. The only regulatory agency in the U.S. requires pyrogenicity studies are the Food and Drug Administration (FDA, 2012).

Pharmaceutical products intended for parenteral use must be free of pyrogens, which can originate from Gram-negative or Gram-positive bacteria, virus and fungi. Exdotoxins

(lipopolysaccharides, LPS) from Gram-negative bacteria are commonly found in parenteral pharmaceutical and medical devices and are found in particular concern to the pharmaceutical industry. Endotoxins are large molecular weight complexes associated with, and shed from, the outer membranes of Gram-negative bacteria. (Westphal, 1975).

Endotoxins consist of three distinct chemical regions: a lipid moiety (lipid A) which is linked to a polysaccharide core that is, in turn, linked to O-antigenic side-chains. Each endotoxin presents a composition and a variable structure that affects its function and biological activity; endotoxin function includes the induction of fever and acute phase proteins, headache and severe hypotensive shock. There is good evidence that the fever response to virus exogenous pyrogens (e.g. endotoxin) is mediated by endogenous pyrogens, i.e. pyrogens generated by the host. Endogenous pyrogens have potent pyrogenic and inflammatory activities and include interleukin 1-Alfa (IL_1Alfa), interleukin-1Beta (IL-1Beta), tumor necrosis factor Alfa (TNF-Alfa) and interleukin-6 (IL-6). Pyrogenic reactions and shock are induced in mammals upon intravenous injection of endotoxin at low concentrations (1 ng/mL) (Fiske, et al., 2001). The maximum level of endotoxin for intravenous applications of pharmaceutical and biologic product is set up to 5 endotoxin units (EU) per kg of body weight per hour by all pharmacopoeias (Daneshiam , et al., 2006).

Physiological effects of pyrogens

The physiological effects of pyrogens in humans are diverse and dose-dependent. First, pyrogen elevate the circulating levels of inflammatory cytokines (e.g., IL-1, IL-6, TNF-Alfa and IL-8) followed by the clinically relevant events of fever, hypotension, lymphopenia, neutrophilia, and elevated levels of plasma cortisol and acute-phase proteins (e.g., C-reactive protein).(Forehand et al., 1989 and Rietschel et al., 1994).

Low doses of pyrogens induce inflammatory reactions without any clinically symptoms, and moderate doses of pyrogens induce fever and changes in plasma composition and high doses of pyrogens can lead to septic shock characterized by cardiovascular dysfunction, including myocardial depression and dilatation, vasodilatation, vasoconstriction, and organ dysfunction (e.g. kidney, liver, lung, or brain) followed by multiple organ failure and death. (Anspach , 2001; Erridge and Bennett-Guerrero , 2002 and Ogikubo , 2004).

TECHINIQUES OF ENDOTOXIN DETERMINATION

The commonly used FDA-approved techniques for endotoxin detection are the rabbit pyrogen test and Limulus Amoebocyte Lysate (LAL) assay (Hoffmann et al., 2005 and Ding and Ho, 2002). The rabbit pyrogen test, developed in the 1920s, involves measuring the rise in temperature of rabbits after intravenous injection of a test solution. Due to its high cost and long turnaround time, the use of the rabbit pyrogen test has diminished, and is now only applied in combination with the LAL test to analyze biological compounds in the

earlier development phase of parenteral devices. Today the most popular endotoxin detection systems are based on LAL, which is derived from the blood of horseshoe crab, *Limulus polyphemus*, and clots upon exposure to endotoxin. The simplest form of LAL assay is the LAL gel-clot assay. When LAL assay is combined with a dilution of the sample containing endotoxin, a gel will be formed proportionally to the endotoxin sensitivity of the given assay. The endotoxin concentration is approximated by continuing to use an assay of less sensitivity until a negative reaction (no observable clot) is obtained. This procedure can require several hours (Daneshiam et al., 2006 and Ding and Ho, 2002). The concentration of 0.5 EU/mL was defined as the threshold between pyrogenic and non-pyrogenic samples (Pinto et al., 2000 and Ding and Ho, 2002).

In addition to the gel-clot technique, manufacturers have also developed two other techniques: turbidimetric LAL technique and the chromogenic LAL technique. These newer techniques are kinetic based, which means they can provide the concentration of endotoxin by extracting the real-time responses of the LAL assay. Turbidimetric LAL assay contains enough coagulogen to form turbidity when cleaved by the clotting enzyme, but not enough to form a clot (Ong et al., 2006). The LAL turbidimetric assay, when compared to the LAL gel-clot assay, gives a more quantitative measurement of endotoxin over a range of concentrations (0.01 EU/mL to 100.0 EU/mL). This assay is based on the turbidity increase due to protein coagulation related to endotoxin concentration in the sample. The optical densities of various test-sample dilutions are measured and correlated to endotoxin concentration helped by a standard curve obtained from samples with known amounts of endotoxin (Sullivan and Watson, 1974). A kinetic chromogenic substrate assay differs from gel-clot and turbidimetric reactions because the coagulogen is partially or completely replaced by a chromogenic substrate (Haishima et al., 2003). When hydrolyzed by the pre-clotting enzyme, the chromogenic substrate releases a yellow-colored substance known as *p*-nitroaniline. The time required to attain the yellow substance is related to the endotoxin concentration (Webster, 1980). However, kinetic turbidimetric and chromogenic tests, although more accurate and faster than the gel-clot, cannot be used for fluids with inherent turbidity such as blood and yellow-tinted liquids, e.g. urine, and their performance may be compromised by any precipitation from solution (Ong et al., 2006). Therefore, different methods for detection of endotoxin in different samples have been studied (Ong et al., 2006 and Poole et al., 2003).

Animal test

A rabbit pyrogen test has been in use since the 1940s. In this test, rabbit are locked in full-body restraints and a test substance is injected into their bloodstream while their body temperature is monitored through rectal probes. The animals can suffer effects which can include fever, breathing problems, organ failure, and fatal shack.

Like all animal based tests the rabbit test is time consuming, costly and give the results that are species specific. However, the rabbit test is scientifically problematic in many additional ways. Even at the highest injected volumes, the detection limit of the rabbit test is above the human fever threshold: humans show a fever response at concentrations as low as 30pg LPS/ml while rabbit's sensitivity varies between 50 and 350pg LPS/ml. In contrast, the human whole blood IL-1 test has a sensitivity of 10pg LPS/ml. In addition, the sensitivity of the rabbit test varies depending on the strain, age and gender of rabbit used.

Other important problems include the fact that the rabbit test often only gives pass/fail rather than a quantitative, answer; that results are influenced by animal distress as well as seasonal variation. Lastly, the rabbit pyrogenicity test doesn't work for many classes of substances including chemotherapeutics, radiopharmaceuticals, certain biological and antibiotics, drugs that cause sedative/analgesics/anesthetics, and vitamins.

Limulus Amoebocyte Lysate (LAL) test

Currently, the method of choice for endotoxin detection in the Limulus Amebocyte Lysate (LAL) test. This test is based on observation that horseshoe carb blood forms colts when exposed to endotoxin (Ding and Ho 2001 and Daneshiam et al., 2006).

Amoebocyte extract form horseshoes carb blood is mixed with a sample suspected of endotoxin contamination, and a reaction is observed if endotoxin are present. The FDA has approved four variations of the LAL test: gel-colt, turbidimetric, colorimetric, and chromogenic assay. The difference in this variation refers to the characteristics of the amoebocyte/endotoxin reaction (e.g. gel-clot produce a precipitate and colorimetric changes color). This test is fast (approx. 30 minutes) and highly sensitive (up to 0.005EU/ml sensitivity).

However, because it only detects LPS endotoxins, some pyrogenic materials can be missed. Also, certain condition (sub-optimal pH conditions or unsuitable cation concentration) can lead to false negatives. Glucans from carbohydrate chromatography matrices can also lead to false positives.

Depyrogenation

Depyrogenation can be defined as the elimination of all pyrogenic substances, including bacterial endotoxin and it is generally achieved by removal or inactivation (FDA, 2010). Depyrogenation, like sterilization, is an absolute term that can only theoretically demonstrate because of test insensitivity. Some scientists regard depyrogenation purely as endotoxin destruction or inactivation, and endotoxin removal as a distinct and unrelated process. Here the former refers to inactivation or destroying an endotoxin present on a component, the latter to the removal of any endotoxin present (Williams, 1995). With depyrogenation inactivation, the total destruction of the “pyroburden” is assumed; with

endotoxin removal it's assumed that a significant portion of the pyroburden has been removed. Other scientists consider both processes to be part of depyrogenation.

Pyrogen removal and inactivation or Destruction

Pyrogen can often be difficult to remove from solution due to the high variability of their molecular weight. Pyrogens are also relatively thermally stable and insensitive to pH changes (Hirayama and Sakata, 2002 and Petsch and Anspach, 2000). However, several techniques exist (McCullough and Novitsky,1985; Weary and Pearson, 1988; Hou and Zaniewski 1990 and Guy, 2003). Because pyrogens are often difficult to remove, inactivation or destruction of the LPS molecules can sometimes be preferable

1- Ion exchange chromatography

Endotoxins are negatively charged, and will bind to an *anion exchanger*. If the target substance is not also negatively charged, it will pass through the column before the endotoxin, and an effective separation can be achieved. This method is sometimes used in the purification of albumins (details follow). Ligands of known affinity to endotoxin can be coupled to an anion exchange system to increase its endotoxin binding strength and further improve the purify of the final product. Typical example of endotoxin binding ligands include histamine, nitrogen-containing heterocyclic compounds, and polymyxin B is known to induce production of interleukin-1, an exogenous pyeogen and thus must be shown to be absent in the final product if used.

An alternative to anion exchange is cation exchange chromatography, in which positively charged solutes bind to the solid chromatography media. In this method, the target binds to the column instead of the endotoxin. The endotoxin then washes through the column, and a pure target is later eluted off the column. Cation exchange chromatography has been shown to effectively purify ß-interferon. (Dembinski et al.,1983).

2-Ultrafiltration

Because the molecular weight of endotoxin is usually over 10 kD, ultrafiltraion can sometimes be used to perform as a size based separation. Due to high variability of endotoxin size, it can be difficult to select the correct membrane, hence this method is best used only when all endotoxin present at larger than 300,000 Da. Commercially available ultra filters have been shown to remove pyrogen to a level below 0.001 EU/ml.

3-Distillation

This method is also based on the large molecular weight and heat stability of endotoxin. Low molecular weight solvents can be easily purified by boiling and collecting the condensed vapor in an endotoxin free vessel. The large LPS molecules do not easily vaporize, and are thus left behind in the heating vessel. This is the method of choice for the purification of water.

4-Reveres Osmosis

Primarily function as a size-excluding filter operation under a highly pressurized condition. It will block 99.5% of endotoxin and ions or salts, but allow water molecules through. USP reverse osmosis (RO) can be used to make water for injection (WFI) (where as to meet the European Pharmacopoeia requirements it can be only be produced by distillation); within Europe it is used to produce highly-purified water.

5-Dilution or rinsing

Preparing sterile products requires manufacturers to control microbial quality. Sterility and endotoxin content are critical because failure to properly manage them can seriously harm, or even kill, patients. Manufacturers use various means to sterilize products, but depyrogenation a process whereby fever-inducing materials are eliminated from materials, components, and equipment—is largely restricted to dry-heat methods or cleaning and rinsing. Dry heat is appropriate for heat-resistant glass or stainless steel items. For other materials, however, depyrogenation is accomplished by cleaning and rinsing surfaces.

Large pieces of equipment such as tanks and permanent piping customarily undergo cleaning in place (CIP). Cleaning requires various chemical agents, including strong acids and bases. Although these agents remove pyrogens, the magnitude of this removal is rarely quantified (James and Agalloco, 2008). The last step in cleaning these items is often a rinse with water for injection (WFI). Samples for cleaning validation, and sometimes for routine verification, may be tested for endotoxins.

Small equipment that cannot be cleaned in place such as filling needles, pumps, and utensils is commonly cleaned with milder agents. Use of these agents ensures the safety of the personnel who perform much of the cleaning process. Small items are sometimes cleaned in parts washers that achieve conditions similar to those in CIP. The final rinse of these materials is always performed with WFI. Depyrogenation of equipment surfaces is not the prime objective of these processes, however. Given the diversity of equipment materials and cleaning processes, expectations for depyrogenation are varied. Some of these processes may achieve depyrogenation effectively; others are less effective.

6-Adsorption

This functions by attracting negatively–charged endotoxin molecule to the carbon bed (e.g, Activated Carbon Beds, where endotoxin is absorbed into charcoal or depth filters). This mechanism is only efficient to a small degree and is affected by a range of environmental factors.

7- **Chemical Sterilization**

Chemical Sterilants as:

Aldehydes – glutaraldehyde, formaldehyde, etc.

Acids – Peracetic, nitric, sulfuric, etc,

Bases – Sodium hydroxide, Potassium hydroxide

Oxygenating compounds – hydrogen peroxide, ozone, chlorine dioxide

Halides – Sodium hypochlorite, chlorine

These must include an aseptic post-cycle quench step to stop process prior to adverse material impact. Acid-base hydrolysis method has been shown to cleave Lipid A from the polysaccharide in the LPS molecule. The lipid moiety alone is not soluble in water. Thus unable to bind to endothelial cells, it is rendered inactive. However, acid-base hydrolysis can denature a target protein, and is thus unsuitable when purifying a protein.

When purifying proteins, sodium hydroxide (NaOH) can be used safely and effectively. It is also widely used for depyrogenation of non-autoclavable equipment (e.g. plastic) and chromatography columns. In fact, when using an anion exchanger or remove pyrogens, it is necessary to clean the column with NaOH after each batch.

Oxidation using hydrogen peroxide is often used as a low cost pyrogen destroying solution. The mechanism for this destruction is unknown, but hydrogen peroxide can easily be removed further downstream in the purification process, and is therefore a useful method of pyrogen removal. However, like acid-base hydrolysis, it is not suitable when purifying proteins. Vapor phase hydrogen peroxide (VPHP) is a relatively new sterilization gaseous agent that is rapidly becoming the gaseous sterilant of choice for many applications, the most well known being the sterilization of barrier isolation systems. Its advantages over other gases, such as ethylene oxide, peracetic acid, chlorine dioxide, and glutaraldehyde, include the following:

1. It does not require temperatures above ambient.

2. There is little or no concern about residual by-products.

8- Gaseous sterilization

The chemically reactive gases ethylene oxide $(CH_2)_2O$, and formaldehyde (methanal, H.CHO) possess broad-spectrum biocidal activity.

• Application: sterilization of re-usable surgical instruments, certain medical, diagnostic and electrical equipment, and the surface sterilization of powders. Ethylene oxide treatment is also considered as an alternative to radiation sterilization in the commercial production of disposable medical devices. EtO is more common internationally than formaldehyde.

Ethylene oxide (EO) treatment is widely used for the sterilization of medical devices. EO acts as a strong alkylating agent who denatures nucleic acids and functional proteins of microorganisms, thereby presenting itself as an excellent sterilization methodology (Mendes et al., 2007). In terms of its disadvantages, EO is flammable and explosive, and EO and its residues are also toxic and carcinogenic (Mendes et al., 2007 and Sílíndír and Özer, 2009).

Function by nucleophilic substitution in the glucosomme of Lipid –A. it is not the most efficient depyrogenation process, and where endotoxin inactivation occurs this is normally a side effect of sterilization.

9-Ionizing radiation (A very slow and inconsistent process)

Sterilization by this method is achieved by exposure of the product to ionizing radiation in the form of gamma radiation from a suitable radioisotopic source (such as cobalt 60 or cesium 137) or of a beam of electrons energized by a suitable electron accelerator. The advantages of the method include low chemical reactivity, low measurable residues, and the fact there are fewer variables to control. Irradiation causes only a minimal rise of temperature, but can affect certain grades of plastics and glass.

10-Moist heat

Conventional autoclaving will not destroy endotoxin. However, the combination of a chemical additive (e.g. hydrogen peroxide) and physical variation (e.g. five hours at 121°C with a pressure of 20 PSI and a pH of 3.8) are sometimes effective. Alternatively, some studies have shown that autoclaving for prolonged periods of times or at higher temperature can be effective at reducing endotoxin and other pyrogen substances (Mosier et al., 1987 and Moesby et al., 2008).

Endotoxin can be inactivated by wet heat, although this is only effective with far lower concentration of endotoxin and is applied to non-heat stable materials. Destruction of endotoxin is far more difficult to achieve, and lower log reductions when compared with dry heat are achieved (Fujii, 2002).

11-Dry heat

Physical destruction, such as convection (transfer of heat by movements of fluid or air), conduction (transfer of heat from adjacent molecules), or irradiation (emission of heat by electromagnetic radiation). Depyrogenation by dry heat for glass in the pharmaceutical industry is the primary endotoxin destruction method used (Nakata, 1993).

This process both sterilizes and depyrogenates and is mainly used for glass components. Dry heat involves subjecting the components to a high level of heat (normally between 180 and 250°C) for a defined time (the higher the temperature, the shorter the time required). The typical cycle is 250°C for not less than 30 minute. For example, the European Pharmacopoeia in chapter 2.6.8 states tow possible time-temperature combinations for dypyrogenation: 60 minutes at 200°C or 30 minutes at 250°C. A quantity of endotoxin destroyed at 250°C for 60 minutes would not necessarily totally destroyed at 200°C at 60 minutes, based on the non-linearity of the thermal destruction curve. Endotoxin destruction at low temperature is of the second-order (Ludwig and Avis, 1990).

Conclusion

Depyrogenation forms part of a critical process in many pharmaceutical production facilities, particularly where glass c\vials and bottles are required for inspecting filling operations.

It should not be forgotten, even achieving successful depyrogenation, that the pyroburden that presents a risk to pharmaceutical is derived from a combination of raw materials, water, active ingredients, environment, and primary packing materials. Risks of endotoxin or other pyrogenic contamination can arise from multiple sources and not simply from the final containers alone. Ultrafiltration of chemicals and buffer solutions, applying appropriates hygienic practices, and performing regular tests can all be helpful.

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