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RESEARCH ARTICLE

REVIEW ON METHODS FOR BIOLOGICAL ASSAYS OF PHARMACEUTICAL PRODUCTS

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ABSTRACT

The present study paper on the biological assays of pharmaceutical product is intended to review on the available literature that are widely applicable in the bioassay of pharmaceutical substances. There is an urgent need for more strategies and procedures for bioassay techniques, including methods that employ multicellular and unicellular organism. The presence of small amounts of pyrogen or endotoxin in recombinant protein preparations and other pharmaceutical product 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 endotoxins from drugs, injectables, and other biotechnological products, an overview of this subject is provided by this paper. An extensive review of literature with regard to methods for pyrogen, endotoxin and toxicity bioassay test of pharmaceutical and biotechnological preparation was carried out. Rabbit pyrogen test is an older, more routine, standard and official in-vivo multicellular test methods in most pharmaceutical compendia. Limulus amebocyte lysate (LAL) is an in-vitro test currently employ in the detection and quantification of endotoxins that are of bacteria and non bacterial origin in a variety of solution, and can be use for screening of starting materials. The possible advantages of LAL test compared to Rabbit method include rapidity, reliability, ease and adaptability. Biological assays are very essential in clinical medicine and other field of science for the role it plays in evaluation and assessment of pharmaceutical and biotechnological preparation.

Key Words: Assay, Bacteria, Rabbit, Multi-cellular, Unicellular.

INTRODUCTION

Since the introduction of the hypodermic syringe in 1855, medicament have been administered parenterally to patient. As early as 1863, the ill effect which follow treatment by injection were being investigated (Antiserum to a mutant, 1982 and Balls and Karcher, 1995). In 1865 Billroth noted that fever appeared in patient while they were receiving a course of injection. Since then, various workers have discovered the causes of fever and the property of the pyrexial agent. In 2012, the United States Pharmacopeia (USP) published a complementary set of three guidance documents on the development, analysis, and validation of biological assays (Chapter, 2012). In this review the term "bioassay**"** is a general name given to any experiment in which the potency or preparation of a drug is measured by its effect on living organism or tissue (Olaniyi and Ogunlana, 1998). Bioassay involves the use of live animals, plant, tissue or cells (in-vitro) to determine the biological activity of a substances such as hormones or drugs (Jeffrey *et al*., 1978). Bioassays are typically conducted to measure the effect of a substance on a living organism and are essential in the development of new drugs, and also in the monitoring of environmental pollutant (Olaniyi and Ogunlana, 1998).

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Development of a drug begin with the selection of prototype of a particular compound of interest (Olaniyi and Ogunlana, 1998), for each area of interest a set of biological test or screening in designed specipically to test for the desired activity (Jeffrey *et al*.,1978). Compound that shows particular good activity in the primary biological screening test such as growth inhibition or promotion are subjected to pathology, toxicology and pharmacology studies to further define their suitability as drugs (Anika and Shetty, 1982). For those that shows promise for clinical use, appropriate formulation are developed. When a compound satisfy all these requirement, a request for IND (investigation new drug application) is made to test the drug on human (Anika and Shetty, 1982; Beckett and Stenlake, 1976; Sandle, 2013). Fever is one of the main symptoms of infectious diseases caused by bacteria, viruses or parasites (Balls and Karcher, 1995). However, the fever reaction is not directly connected to live microorganisms, at the end of the 19th century, fever-inducing contaminations were found to be heat-stable. Soon afterwards, the connection was established between pyrogenicity and the heat-stable endotoxin originating from Gram-negative bacteria (Rietschel *et al*., 1994; Sandle, 2013). Because the mechanisms of fever generation were unknown at that time, the fever-causing materials were classified as either exogenous or endogenous pyrogens. Exogenous pyrogens are materials from the environment (for example, debris from microorganisms) that cause fever in mammals. Until now, endotoxins, a group of chemically similar cell-wall structures of Gram negative

bacteria, are the best characterised exogenous pyrogens, and these have been shown to be lipopolysaccharides (Eperon and Jungi, 1996; DeGroote *et al*., 1997).

Justification for the Review

Phamaceutical industries try to ensure the production of excellent quality drugs by employing various methods of control and regulation of the chemicals and other antimicrobial property of the product before channeling to consumers (Beckett and Stenlake, 1976).This is in accordance with the world health organization provision (WHO, 1986), as specified by the British and United state pharmacopeias (BP, 1998; Chapter, 2012). In the biotechnology industry, Gram-negative bacteria are widely used to produce recombinant DNA products such as peptides and proteins. Many recombinant proteins are produced by the Gram-negative bacteria *Escherichia coli*. These products are always contaminated with endotoxins (Hirayama and Sakata, 2002). For this reason, proteins prepared from Gram-negative bacteria must be as free as possible of endotoxin in order not to induce side effects when administered to animals or humans (Antiserum, 1982).

Objectives

The aim of the present work is to review on the available literature that are widely applicable in the bioassay of pharmaceuticals product such as drugs, syringes, needles or substances suspected as having the potential of use as chemotherapeutic agents.

This is with the following objectives:

- Developing an overview about the available reasons for bioassay.
- Strategies and procedures involves in the several techniques available
- Review some of the methods of bioassay that are applicable in vitro and in vivo
- Identify the best and novel bioassay methods available for injectable pharmaceutical substances.

Types of bioassays

Bioassays are classified based on grade as well as quantal effect (Olaniyi and Ogunlana, 1998) .

Quantal

A quantal assay involves an "all or none response". For example: Insulin induced hypoglycemic convulsive reaction or the cardiac arrest caused by digitalis. The response is either +ve or -ve, there is no intermediate response e.g.—either convulsion occurs or doesn't occur; similarly is with cardiac arrest. In case of toxicity studies, the animal receiving a dose of drug either dies or does not die. Also, no intermediate response is possible (Friberger, 1985; Olaniyi and Ogunlana, 1998).

Graded

Graded assays are based on the observation that there is a proportionate increase in the observed response following an increase in the concentration or dose. The parameters employed in such bioassays are based on the nature of the effect the substance is expected to produce. For example: contraction of smooth muscle preparation for assaying histamine or the study of blood pressure response in case of adrenaline .A graded bioassay can be performed by employing any of the below-mentioned techniques (Friberger, 1985; Olaniyi and Ogunlana, 1998).

The choice of procedure depends on:

- The precision of the assay required
- The quantity of the sample substance available
- The availability of the experimental animals.

Techniques

- Matching Bioassay
- Interpolation Method
- Bracketing Method
- Multiple Point Bioassay (i.e.-Three-point, Four-point and Six Point Bioassay)
- Divided bioassy

Pyrogen test

A pyrogen is a protein that can induce fever in a patient by triggering a series of immune reaction. Absence of pyrogen is a critical safety precaution for all drugs administered parenterally (Booth, 1986; El-Khalik and Benoliel, 1982). Burdon –Sanderson in 1876 conducted a research and found that a molecule small enough to pass through diatomite bacteria proof filters which is of bacterial origin , he then finally called it" pyrogen" Seibert in 1923 found that pyrogen were produce by water born bacteria and correct distillation would eliminate these substances. More recently Co Tui and Schrift in 1942 suggested that air born bacteria as well as some yeast and mould, also produce pyrogen.

Wagner and Bennett in 1950 found that viruses too caused pyrogenic effect. The pharmacopoeial test for pyrogens are limit test performed on rabbits and are describe in the british pharmacopoeia in 1968,the united state pharmacopoeia in 1955 and the international pharmacopoeia (BP, 1998; Chapter, 2012). The samples to be tested are large compared with the amount normally injected in to a patient, further the rabbit is more sensitive to pyrogen than humans and subsequently these pharmacopoeial test are satisfactorally for the detection of pyrogen. Charlton in 1965 described pyrogen testing for radiopharmaceutical s substances and suggested that product can be classified in to two general classes:

- **High risk**, which include
  - Product administered in large volume
  - Product which are good media for media for microbial growth.
- **Low risk**, which include
  - Product administered in small volume.
  - product which either contain substance inhibitory to bacterial growth.

The pyrogen test is designed to limit the risk febrile reaction to an acceptable level in the patient to the administration by injection of the pharmaceutical product (Booth, 1986; El-Khalik and Benoliel, 1982; Greisman and Hornick, 1969).

There are two method to ascertain that a product is non-pyrogenic. These are:

- Conventional Rabbit pyrogen test.
- Limulus amebocyte lysate (LAL) test

Conventional Rabbit pyrogen test

In this report, the term "rabbit pyrogen test" refers to an animal test using rabbit as defined in the pharmacopoeias (BP, 1998; Chapter, 2012). The presence of pyrogens leads to an increase in the body temperature of rabbits. Contamination with pyrogens is hazardous, especially in drugs for intravenous use. Since numerous exogenous pyrogens are heat-stable, sterilisation is not sufficient to exclude fever reactions. In the 1940s, the rabbit pyrogen test was introduced as an analytical control procedure for the detection of fever-causing contamination, and was consequently incorporated into various pharmacopoeias and guidelines (Bellentani, 1982; El-Khalik and Benoliel, 1982 and Greisman and Hornick, 1969). However, the rabbit pyrogen test has several drawbacks. The sensitivity of rabbits toward endotoxin reference preparations depends on the strain used and the experimental conditions (for example, age, gender and housing conditions (van Dijck and van de Voorde, 1977). Nevertheless, the rabbit strain to be used is not defined in the respective monographs of the pharmacopoeias.

Even if the highest permitted volume (10ml/kg body weight) is injected, the detection limit is restricted to 50–350pg (i.e., 0.5–3.5IU) of LPS/kg; however, the human fever threshold is around 30pg/ml (Bonenberger *et al*., 2000 and Greisman and Hornick, 1969), for many drugs, the volume tested is significantly smaller. Drugs that are to be intravenously injected must be shown to be of a pyrogen-free .For other parenteral drugs, given subcutaneously or intramuscularly in much smaller 100 T. (Hartung *et al*. volumes) (for example, vaccines), a maximal acceptable endotoxin concentration has to be defined for quality-control purposes (Hartung and Wendel, 1996; Hartung *et al*., 2000; Hartung and Seeger, 1994) . However, the rabbit pyrogen test is not suitable for the control of such a limit since it is not a quantitative test, i.e. it gives only a pass/fail result, and it is less-well standardised (Bellentani, 1982; Hull *et al*., 1993).

Rabbit pyrogen test is a simple test which involves the measuring of the rise in temperature of rabbit following the intravenous infection of a test solution and is designed for product that can be tolerated by the test rabbit in a dose not to exceeded 10ml per kg injected intravenously within a period that require preliminary preparation or are subjected to special condition of administration, it is important to follows the additional direction given under each monograph or, in the case of antibiotics or biological, or the additional direction given in the respective official compendia (Olaniyi and Ogunlana, 1998) .

Apparatus and Diluents

The syringes, needles, and glassware must be rendered free from pyrogens by heating at 150^{o}c for not less than 30 minute or by any other suitable methods. This is followed by heating all diluents and solution for washing and rinsing of devices or parenteral injection assembles in a manner that will assure that they are sterile and pyrogen test on representative portion of the diluents and solution for washing or rinsing of the apparatus. Where sodium chloride is specified as a diluents, it is important to use injection containing 0.9% of Nacl (Olaniyi and Ogunlana, 1998) .

Temperature Recoding

One must employ an accurate temperature – sensing device such as a clinical clinical thermometer, or thermistor probe or similar probe that have been calibrated to assure an accuracy of + - 0.1 and have been tested to determined that a maximum recording is reached in less than 5 minutes. This is followed by inserting the temperature- sensing probe in to the rectum of the test rabbit to a depth of not less than 7.5cm, and after a period of time not less that previously determine as sufficient, the rabbit's body temperature is recorded (Olaniyi and Ogunlana, 1998) .

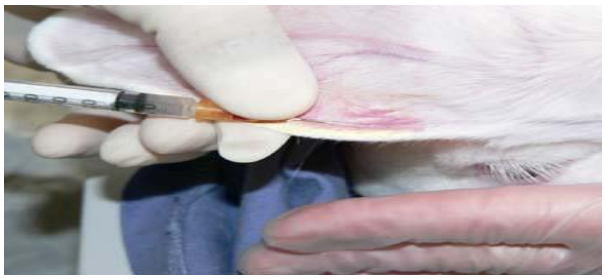
Method of analysis

Use healthy, adult rabbit preparabily of the same variety, house the animals individually in an area designed for pyrogen test (area of uniform temp. 2 and uniform humidity and free from disturbance likely to excite them, the animals are given ad libitum water and food commonly use for laboratory animals. Access to water may be allowed, the animal may be place under the condition of the test atleast 1hr before the injection. Take two temperature measurement of each animal at an interval of 30min. The mean of the two temperatures serve as "control temperature" of the animal. The control temperature recorded for each rabbit constitute the temperature from which any rise following the injection of the materials is calculated. Those animal for which the two temperature have deviate by more than 0.2 from the mean should not be use in the test, nor should any animal with control temperature below 38.0 or below 39.8 (Olaniyi and Ogunlana, 1998).

Render the syringe needles and glass ware free from pyrogen by heating at 250 for 30minute.Inject 10ml per kg body weight of the test solution in to a margin vein of the ear of each of the 3 rabbit, when injection has been complete, record the temperature of the animal during the period of 3hrs,taking the measurement every 30min using an accurate thermometer graduated in 0.1 by inserting the temperature sensing device in to the rectum of the test rabbit depth of about 6cm (Greisman and Hornick, 1969; Kundsin and Walter, 1980).

Limitation of Rabbit pyrogen test

- Radiopharmaceutical substance cannot be tested using rabbit methods
- Expected gap between the observed pyrogenicity in rabbits and pyrogenicity in humans due to species differences.
- Its expensive and not quantitative
- Its not suitable for some product such as prostaglandins, anticancer agent.



Rabbit ear injection

Limulus amebocyte lysate (LAL)

In the 1970s, radiopharmaceutical drugs were introduced into clinical practice. These drugs cannot reasonably be tested in rabbits.The solution was the LAL test (Cohen *et al*., 1986; Cooper, 1979 and Cooper *et al*., 1972) which is over the last 30 years, has replaced the rabbit pyrogen test for these and a number of other products (Liebsch, 1995). Limulus amebocyte lysate (LAL) is an aqueous extract of blood cells (amoebocytes) from the horseshoe crab, *Limulus polyphemus*. LAL reacts with bacterial endotoxin or lipopolysaccharide (LPS), which is a membrane component of Gram negative bacteria. A single *Escherichia coli* contains about 2 million LPS molecules per cell. Endotoxin elicits a wide variety of pathophysiological effects. This reaction is the basis of the LAL test, which isvc the used for the detection and quantification of bacterial endotoxins. It is produce by associate of cape cod, incoperated, and sold under their registered trade name 'pyrotell' in the U.S.A. (Cohen *et al*., 1986; Duff and Atkins, 1982 and Flint, 1994)

Horseshoe crab anatomy

Horseshoe crab blood

The presence of endotoxin results in the clotting reaction of the amoebocyte lysate of horseshoe crabs. The test is historically referred to as the *Limulus* amoebocyte lysate (LAL) test, although lysates from both *Limulus polyphemus* and *Tachypleus tridentatus* are employed products (Booth, 1986; Flint, 1994; Krüger, 1981 and Liebsch, 1995). The LAL reaction is primarily based on the clotting reaction of the haemolymph of the horseshoe crab in the presence of bacterial endotoxins.

There are various approaches for measuring the LPS-induced reaction,for example, clotting reaction, kinetic turbidimetric measurement, chromogenic endpoint and kinetic reaction (Gardi and Arpagaus, 1980), which show some dissimilarities . This test is specific for endotoxins from Gram-negative bacteria, and precisely because of this property does not detect pyrogenic substances other than endotoxins. Nevertheless, in most cases, endotoxins represent the leading pyrogenic contamination, so it has generally been possible to assure "pyrogen-free" products with the LAL test (Mc Cullough and Weidner-Loeven, 1992; Opal *et al*., 2007).

The investigation of the role of endotoxin, also known as lipopolysaccharide (LPS), in the pathophysiology of sepsis in patients who are treated in hospitals has been pursued since LPS was first discovered in the 1800s as a Gram-negative cell wall toxin capable of triggering lethal shock. The role of bacterial LPSs in human disease has been reviewed by Opal (Opal *et al*., 2007), Munford (Munford, 2008) and Danner (Munford, 2008). Zeigler and colleagues were among the first to attempt anti-endotoxin therapy in patients with severe sepsis using anti-serum against the J5 mutant of *Escherichia coli* as a source of anti-core glycolipid antibody (Ziegler, ?). There is a clear need for a new method for the detection of non-endotoxin pyrogens *in vitro*. No test means there are no data, but does not mean there are no cases (Chapter, 2012)

Possible Advantage of LAL test compared to United State Pharmacopoeia (USP) Rabbit Test

- The LAL test is an in vitro test which require minimal number of items to complete the test offers, rapidity and reliability.
- The ease and adaptability of the LAL test will allow it to be use in many different situation for which application of the rabbit test would be impossible. Examples of such case include many product administered parenterally such as prostaglandin, anticancer agent and etc.
- Its more acceptable, specific, rapid and sensitive methods for endotoxin assay of parenteral drugs.
- Provide less operational time and cost.

Limitation of Limulus amebocyte lysate (LAL) test

- LAL test Cannot detect non-endotoxin pyrogens
- Some biological product cannot be tested using LAL test
- There is expected gap between the observed pyrogenicity in LAL and pyrogenicity in humans.

The Need for Novel Pyrogen Tests

In this report, the term "novel pyrogen test" refers to systems that can detect endotoxins ECVAM Workshop 43: novel pyrogen tests 101 and other pyrogens *in vitro* by measuring the release of fever-inducing mediators than the LAL test (Balls and Karcher, 1995; Rietschel *et al*., 1994).The most important difference between the rabbit pyrogen test and the LAL test is the failure of the latter to detect non-endotoxin pyrogens. On the other hand, the BET makes it possible to test the pyrogenicity of products that have not been found to be testable in the rabbit, for example, radiopharmaceuticals. The recently established, more sophisticated, kinetic and colorimetic BET systems have increased sensitivity. Nevertheless, there remain a huge number of complex

preparations, such as biologicals, which cannot be tested with BET systems (Balls and Karcher, 1995). Therefore, initially, a novel pyrogen test should combine the advantages of the rabbit pyrogen test and the BET, without suffering from their disadvantages. It should be robust, quantitative, sensitive, applicable to a wide variety of products, and it should detect nonendotoxin pyrogens. The limitations of the rabbit pyrogen test and the BET systems demand the establishment of advanced pyrogen test methods, which should meet the following specifications (Rietschel *et al*., 1994; Lane *et al*., 1985):

- They should not involve animals;
- They should mimic the human fever reaction;
- They should detect a broad range of pyrogens;
- They should be widely applicable (minimal interference with the products to be tested);
- They should be quantitative or semiquantitative; and
- They should be simple and practicable (the reagents should be available in less-well-equipped

Current Status of Novel Pyrogen Tests

In recent years, a number of new approaches to pyrogen testing have been reported, which are mainly based on the use of human cells. Pyrogens induce the release of mediators that can be quantified by immunochemical methods. The three main approaches involve the use of cells of various origin (Rietschel *et al*., 1994):

- Leucocyte cell lines,
- Isolated primary blood and
- Human whole blood.

Novel pyrogen tests based on leucocyte cell lines

Principle

Human monocyte/macrophage-like cell lines, such as MonoMac-6 and THP-1, or clones derived from such lines and murine macrophage-like cells, such as RAW264.7, have been widely used for pyrogen testing. Pyrogens induce cytokine production (IL-6, TNF-α) or lead to the formation of metabolites (neopterin, nitrite) from cytokine-inducible pathways cells, which can then be measured in the supernatants of the cultured cells by ELISA methods (IL-6, TNF-α, neopterin) or detected spectrophotometrically through the Griess reaction (Balls and Karcher, 1995; Mc Cullough and Weidner-Loeven, 1992).

Examples

MonoMac-6. MonoMac-6 cells () are incubated overnight in Iscove's modified Dulbecco's medium (IMDM) supplemented with Glutamax-1, 2% fetal calf serum (FCS) and 100IU penicillin/streptomycin, in 96-well, flatbottomed, tissue culture plates. Commercially available ELISA kits are used for detection of IL-6 or TNF-α. The test system has been compared with the rabbit pyrogen test (specific pathogen-free rabbits) and the LAL test (gel clot); the sensitivity was 0.125IU/ml for Endotoxin Standard Biological Reference Preparation Batch No. 2 *(European Pharmacopoiea [Ph. Eur.]*) compared with 0.03IU/ml for LAL.

The limit of detection for LPS is 10pg/ml in the culture medium, and the absolute detection limit is 100pg/ml LPS. The test system shows a 100% LPS spike recovery rate in albumin. The presence of immunoglobulin G inhibits IL-6 and TNF- detection (dilutions should be tested) (Taktak *et al*., 1991). For some bacterial vaccines, as shown for typhoid, pneumococcal and meningococcal polysaccharides in a pilot evaluation, the LAL test can be combined with the MonoMac-6 test. *THP-1 and RAW264.7*. THP-1 or RAW264.7 cells (Hartung *et al*., 2000) are incubated overnight in 96- 102 T. Hartung *et al*. well plates, in serum-free culture medium (Ultraculture for THP-1, X-Vivo for RAW264.7, both from BioWhittaker (Verviers, Belgium) (Peterbauer *et al*., 1999). In order to increase their sensitivity, the cells are co-stimulated with 250IU/ml of human and 50IU/ml of pyrogen free murine recombinant Interferon-γ, respectively (Werner and Wachter, 1995; Werner-Felmayer, 1999 and Werner-Felmayer, 2000). For THP-1 cells, the induction of GTP cyclohydrolase-I is determined by measuring neopterin by ELISA, whereas for RAW264.7 cells, the induction of NO synthase is determined by measuring nitrite derived from NO by reaction with molecular oxygen (the Griess reaction, OD at 540nm).

Comparison with other test systems indicates that, for pyrogens from Gram-negative bacteria, the test systems correlate well with LAL test results, whereas they are superior for detection of pyrogens from Gram-positive bacteria. Correlation with results from the rabbit test and another cell-culture-based test (detection of TNF-α formed by THP-1 1G3 cells) could be demonstrated for a limited number of samples (Eperon and Jungi, 1996); however, further, more-detailed, studies are needed. The limit of detection for LPS is 1–10pg/ml (depending on the LPS preparation), and pyrogens from both Gram negative and Gram-positive bacteria are detected. *Sub clones derived from the human monocytoid THP-1 and MonoMac-6 cells.* Sub clones derived from the human monocytoid THP-1 and MonoMac-6 cells have been selected on the basis of their high sensitivity to endotoxin, and were shown to be phenotypically stable (DeGroote *et al*., 1997). The cells are primed with calcitriol for 44 hours, followed by a 4-hour exposure to the test sample. Cell culture supernatants are collected and TNF-α is deter - mined by ELISA. Comparison with the LAL test shows a good correlation, and the tests are of comparable sensitivity; however, the cell culture method appears to be more specific. The limit of detection for LPS was 15pg/ml, i.e. 0.15IU/ml, for LPS from *Escherichia coli* O55:B5. Robustness and day-to-day variability are under further evaluation (Eperon and Jungi, 1996; Mc Cullough and Weidner-Loeven, 1992 and Meisel, 1995).

Tests based on isolated primary blood leucocytes

Monocytes and lymphocytes are isolated from blood samples by density-gradient centrifugation, then the washed cells are incubated in FCS-containing medium and exposed to the test sample for several hours (Duff and Atkins, 1982). The production of IL-1, TNF-α or IL-6 is then measured by immunochemical methods (for example, an ELISA or a bioassay). Data on comparison with other test systems are not available. The limit of detection is about 10pg/ml for LPS. The test systems are considered to be laborious and variable, due to the various stages in cell preparation. Nowadays, better-standardised and more-easily handled test systems based on

cell lines or whole blood are available, and most of the tests involving isolated primary blood leucocytes have not been further developed or standardised. However, some such test systems are still in use, especially in industry (El-Khalik and Benoliel, 1982; Kazatchkine, 1990)

Tests based on human whole blood

Principle

Fresh human whole blood is diluted and incubated in the presence of the sample to be tested. The production of IL-1□, IL-6, TNF-□ or prostaglandin E2 (PGE2), as measured by using immunochemical methods, correlates with the content of pyrogens in the sample (Fennrich *et al*., 1998). The blood can be used for up to 8 hours after withdrawal, and the use of cryopreserved blood is also possible (DeGroote *et al*., 1997; Fennrich *et al*., 1998)

Examples

Human whole-blood model (endpoint IL-1b). Human whole blood (10% v/v in saline) and the test sample are incubated overnight, and IL-1 is subsequently measured in the supernatant by ELISA. A simplified one-plate assay is under development. Comparison with other systems reveals a good correlation with the rabbit pyrogen test, whereas differences are evident between the potencies of LPS derived from different bacterial species as estimated by the LAL test and the human whole-blood model. The limit of detection is <50pg LPS/ml sample (4.2pg/ml final concentration). The donor-dependent variation in the threshold of IL-1β release is low. The test system is not inhibited by up to 10% dimethyl sulphoxide; however, cryopreserved blood gives a much stronger IL-1β response to endotoxin. *Human whole-blood model (endpoint IL-6*) (DeGroote *et al*., 1997).

Human whole blood (10–20% v/v in saline) and the test sample are incubated for 4 hours, and IL-6 release is measured by ELISA. A rapid one-plate pyrogen test has been developed, which combines the incubation phase with the evaluation phase in a 96-well plate, which is coated with affinity-purified monoclonal anti- 104 T. Hartung *et al*. bodies against IL-6. This one-step incubation and measurement of IL-6 increases the sensitivity; thus, the limit of detection is 0.03IU/ml, i.e. 3pg/ml LPS, for the one-plate assay, and 0.06IU/ml, i.e. 6 pg/ml LPS, for the two-plate assay (DeGroote *et al*., 1997; Kazatchkine, 1990).

Acceptable standard for the bioassay methods quoted

Positive reaction in the rabbit pyrogen test

A positive result in the rabbit pyrogen test demonstrates contamination with pyrogens according to the requirements and definitions of relevant monographs and guidelines. However, there remains a gap between the observed pyrogenicity in rabbits and the expected pyrogenicity in humans. Due to species differences, some materials (for example, biological for human use) might cause fever in humans but not in rabbits, and vice versa. Any temperature increase below the control temperature are considered as zero rise, if no rabbit show an individual rise in temperature of 0.6 or above its respective control temperature and if the sum of the 3-individual maximum temperature rises does not exceed 1.4, the product meet the requirement for the absence of pyrogen, if 1 or2 rabbit shows a temperature rise of 0.6 or more ,or if the sum of the temperature rises exceed 1.4,continue the test using 5 other rabbit ,if not more than 3-of the eight show individual rise in temperature of material o.6 or more and if the sum of the eight temperature rise does not exceed 3.7, the test material meet the requirement for the absence of pyrogen (Olaniyi and Ogunlana, 1998; WHO, 1986). A positive result in the LAL test is sufficient proof that the product is pyrogenic in humans. Nevertheless, a negative result does not mean that the product is pyrogen-free, since nonendotoxin pyrogens are not detected with the LAL test (Hull *et al*., 1993; Lane *et al*., 1985; Poole and Mussett, 1989 and Poole *et al*., 1997).

It can be expected that the LAL test will be several-fold more sensitive than the average rabbit pyrogen test. Judgement and clinical experiences when available, will have to be use to established satisfactorally level of pyrogenicity detectable by the limulus amebocyte lysate test in product that yield a positive LAL test when tested, undiluted but are negative in a rabbit pyrogen test .Ideally, the quality of any drug or device when tested by the LAL test should be such that it will yield negative LAL test when the test is perform on an undiluted sample of the product. Because performance of the LAL test is subjected to a certain amount of variability depending upon the skills of the operator and quality of the reagent and equipment used (Olaniyi and Ogunlana, 1998; Poole and Mussett, 1989; Poole *et al*., 1997).

Conclusion

Pharmaceutical product are always preparation most likely to be contaminated with pyrogen, these product can reasonably be tested using several techniques as it has been seen, the novel pyrogen test using human blood has replaced the limulus amoebocyte lysate methods and rabbit united state pharmacopeias method as it combine their advantage without suffering from their disadvantage.

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