THE ENDOTOXIN CRITERIA DOCUMENT: ENVIRONMENTAL MONITORING FOR ENDOTOXIN AEROSOLS

R.R. Jacobs
Department of Environmental Health Sciences,
University of Alabama at Birmingham
Birmingham, AL

Abstract

In 1978, the OSHA cotton dust standard specified the use of the vertical elutriator (VE) for measuring airborne respirable dust. Efforts to define the etiology of byssinosis resulted in the analysis of VE dust filters for endotoxin. This effort has served as the basis for developing methods to evaluate different environments for endotoxin. The basic procedure consists of collection of a airborne gravimetric sample which is extracted with a pyrogen-free diluent and assayed by the LAL. However, there are no established guidelines for the sampling and analysis of aerosols or bulk samples for environmental endotoxin. This report reviews the methods that have been used to evaluate environmental endotoxin and makes recommendations regarding sample collection, storage, and analysis.

Introduction

The collection of aerosols for endotoxin analysis is a relatively recent effort which is based on studies to understand the etiology of byssinosis, a lung disease caused by exposure to cotton dust. Based on previous studies, the 1978 cotton dust standard, promulgated by the United States Occupational Safety and Health Administration (OSHA), required that gravimetric dust samples be collected with the vertical elutriator (VE). The VE is an area sampler with a nominal particle size cutpoint of 15 µm. Efforts to define a more specific the etiology of byssinosis resulted in the analysis of VE dust filters for endotoxin. For the analysis, the filter was weighed, extracted with pyrogen free water (PFW), and assayed for endotoxin by the LAL. The results were expressed either as a weight of endotoxin per weight of dust (µg/mg) or as a weigh of endotoxin per cubic meter of air (µg/m^3). Based on these types of analysis, both Rylander et. al. (1985) and Castellan et. al. (1987) demonstrated a much stronger correlation of airborne endotoxin with an acute pre to post exposure change in the forced expiratory change in one second (FEV_1) than was observed for gravimetric dust and FEV_1. These studies have served as the basis for evaluating other environments for airborne endotoxin.

The basic procedure that has been used in all studies published to date for the evaluation aerosols for endotoxin include variations of the following steps:

1. Collection of a gravimetric dust sample
2. Preserve the sample for storage prior to extraction
3. Extraction of the sample for analysis
4. Storage of the extract prior to analysis
5. Analysis of the extract by the LAL

1. Collection of a Gravimetric Dust Sample:

Important variables in the collection of an airborne samples for endotoxin analysis include the following considerations: 1. Area vs personal sampling; 2. Particle size selection; 3. Sampling time; and 4. Filter type, pore size, and diameter. Based on data from published studies, it appears it will be necessary to tailor the sample collection media for the specific aerosol of interest. However, the impact of these variables on the measurement of endotoxin is not discussed in this paper.

2. Sample Preservation And Storage Prior to Extraction:

There have been no published studies that have evaluated the stability of airborne endotoxin under different storage conditions prior to filter extraction. Chun et. al. reported that the levels of endotoxin in baled cotton stored in a warehouse exhibited a trend of gradual decline over a 10 year period, but that there was no difference between any two successive years (1992). This study also demonstrated that the levels of GNB decreased more rapidly that the levels of endotoxin, however, the declines were also gradual. The major consideration when storing either bulk or airborne filter samples is to prevent the growth of additional microorganisms that will contribute to the endotoxin or LAL reactive material load of the sample. Therefore, it is recommended that samples be handled aseptically and stored either desiccated or at 4 degrees C to prevent growth of GNB.

3. Extraction of the Sample for Analysis

The extraction method most frequently used is to suspend the filters in a diluent of either PFW, PFW with surfactant (0.05% Tween 20 (Douwes et. al., 1995)) or pyrogen free buffer (phosphate triethylamine (Milton et. al., 1990)). The sample is then either shaken or sonicated at room temperature for specific times, ranging from 10-120 minutes. If the extracted sample contains suspended particulates it is generally centrifuged at 1000xg for 10 minutes and the supernatant used for the LAL analysis. Although most studies have used PFW there is not a consensus regarding the most effective method for extraction. Additional studies are needed to determine the composition of the optimum extract for airborne samples.

4. Sample Storage after Extraction:

Storage Container: In a study evaluating the recovery of aqueous preparations of E coli 0113 LPS placed in different types of containers, the highest recoveries were observed from polystyrene containers. For these studies, the LPS samples were placed in the container, dried and reconstituting in PFW. Polypropylene yielded the lowest

Reprinted from the *Proceedings of the Beltwide Cotton Conference*
Volume 1:156-157 (1997)
National Cotton Council, Memphis TN

recoveries. Recoveries from flint glass and borosilicate glass were variable and depended on the type of endotoxin preparation(Novitski et. al., 1986). Douwes et. al. evaluated the recovery of endotoxin from borosilicate glass, soft glass culture tubes, and polypropylene tubes and observed no container effects for samples store up to a year at 7 C (1995).

Storage Conditions: Four studies have reported on the effects of freezing on the endotoxin stability. Olenchock et. al. reported that endotoxin levels in samples of organic dust frozen at -85°C were stable over a 30 day period (1989). The samples in this study were repeatedly frozen at -85°C and thawed. Chun reported that repeated freezing (-78°C) and thawing resulted in a progressive decrease in endotoxin levels in cotton dust samples (Chun and Perkins., 1994). Millner reported that endotoxin levels were reduced to half of the original level after being stored at -20°C (Millner et. al., 1988). Douwes et. al. reported that preparation of commercially available LPS in pyrogen free water and stored at -20 C lost 25% of the LAL activity after each freeze thaw cycle (1995). But samples stored at -20 for up to a year did not lose activity. A 20% decrease was observed after freezing and thawing house dust samples stored at -20C. This study also reported that LPS stored in PFW at 7 C was stable for up to one year. Based on these studies it is recommended that samples be analyzed without freezing after extraction. If it is necessary to freeze a sample prior to analysis it should be stored at -70 to -85C and after thawing for analysis discarded. Repeated freezing or thawing should be avoided.

5. Analysis of the Extract by LAL

A variety of tests have been developed for measuring the presence or absence of endotoxin. The methods can be divided into two broad categories, analytical assays that measure the total amount of endotoxin in a sample and biological assays that measure the portion of endotoxin that is biologically available to the specific assay. "Biologically available endotoxin" refers to that portion of the "total endotoxin" in a sample that is active in a given bioassay. The quantity of biologically available endotoxin may differ for different bioassays and is less that the total endotoxin in a sample. Two additional terms used to describe environmental endotoxin are "bound" and "free" endotoxin. Bound endotoxin refers to that portion of either biologically available or total endotoxin associated with viable or dead bacterial cells and free endotoxin refers to that portion that occurs as isolated endotoxin, LPS, or Lipid A (Rylander, 1987).

Analytical Methods: A variety of instrumental techniques have been used to evaluate the chemical composition of LPS and recent studies have attempted to use specific constituents of LPS as biochemical markers for quantifying environmental endotoxin. Specifically, 3-hydroxy fatty acid, a LPS-specific component has been used to evaluate environmental endotoxin (Sonesson et. al., 1990). The 3-hydroxy fatty acid was evaluated in suspensions of *E. coli* by GC-MS and resulted in LPS levels approximately 10 times higher than that measured by a bioassay (LAL assay). The different results reflect the differences in measuring total endotoxin and biologically available endotoxin.

Biologically Based Methods:

Immunologic Methods: Monoclonal antibodies have been developed against the lipid A component of LPS (Bogard et. al., 1985). However, the use of these antibodies to quantify endotoxin have not been successful (Millner, 1989). This may be related to the availability of the Lipid A moiety for reactivity with the antibody.

The Rabbit Pyrogen Test: In the United States, the necessity for having a reliable pyrogen test led to a collaboration between the US Food and Drug Administration (FDA), the National Institutes of Health (NIH), and drug companies to develop a valid pyrogen test. The result was the adoption of the Rabbit Pyrogen Test (RPT) as the official pyrogen test in 1945.

The RPT test involves measuring the rise in body temperature of rabbits after intravenous injection of a test product and is intended to limit to the risk of febrile reaction from a patient caused by use of medical products (Weiss, 1978). The test relies on the fact that the minimum pyrogenic dose on a per kilogram basis is the same for rabbits and man (Greisman et. al., 1969). The test is cumbersome and involved. Using the RPT it would be very difficult to test the number of samples generated in an epidemiological survey. Historically, the test has been used to provide a pass or fail determination for endotoxin contamination for a product. The test has generally not been used to attempt to quantify the amount of endotoxin present in solutions (Weary and Wallin, 1973).

Limulus Amebocyte Lysate Assay: LPS can be measured using the Limulus Amebocyte Lysate (LAL) test. The LAL test is derived from the hemolymph of the horseshoe crab, Limulus polyphemus. Hemolymph is composed of plasma which contains the oxygen carrying protein hemocyanin and a cell called the amebocyte. When the horseshoe crab is injured the cells lose their ovoid shape becoming multilobulated, or "ameboid", aggregate at the site of injury, degranulate and form a protective clot. This coagulation process occurs when Limulus sp. is exposed to LPS of GNB (Levin, 1987). Based on these observations a sensitive test for detecting the presence of LPS was developed. The rate of activation of the coagulation cascade is directly related to the concentration of endotoxin (Levin and Bang, 1968).

Endotoxin Standards: Results of endotoxin analysis by the LAL assay have historically been reported in units of weight/volume (ng/ml or nm/m^3) or weight/weight (ng/mg). However, marked differences have been observed in LAL reactivity to different LPS preparations making interlaboratory comparisons of endotoxin analysis difficult.

Additionally, different batches of Limulus lysate respond differently to the same preparation of endotoxin. To overcome these difficulties, the United States Food and Drug Administration (FDA) developed a "Reference Standard Endotoxin" (RSE) from *E. coli* 0113:H10K. The FDA has required that all LAL manufactures label the sensitivity each specific batch of Limulus lysate relative to EC-5 RSE. The use of EUs provides a common basis for comparison between laboratories. This strategy was developed primarily for pyrogen testing for the pharmaceutical industry and Popendof et. al. (1986) has recommended that EUs be used for evaluating environmental samples.

Assay types: Gel-clot Test: By the gel-clot method is based on the ability of very minute quantities of endotoxin to induce gelation in LAL (Levin and Bang, 1968; Sullivan et. al.,1976; Seigel and Nachum, 1977). The test is typically performed by mixing equal volumes of LAL and test solution in a depyrogenated glass tube, mixing gently, and incubating at 37 C for 1 hour (Seigel and Nachum, 1977). At the end of incubation the tube is gently inverted (180°) (Seigel and Nachum, 1977). If a solid gel remains in the tube a positive reaction is said to have occurred (Seigel and Nachum, 1977). The test estimates the minimum concentration of endotoxin which will cause the production of a firm clot in the LAL reagent. The test has been shown to be reproducible, accurate, and capable of detecting quantities of LPS in the range of 1000 to 100 pg/ml. It is faster, less costly, and more sensitive (3-300x) than the USP rabbit pyrogen test (Wachtel and Tsuji, 1977), and requires less instrumentation than either the turbidimetric or chromogenic assay techniques (Berzofsky, 1983).

However, the test has several limitations: 1) It is semiquantitative in nature; 2) It is prone to false negatives--If the reaction is disturbed during the test a clot may not form; 3) The test is highly sensitive to technique; 4) The test suffers high assay variability--Coefficients of variation of 50% are accepted as normal assay variability; 5) Because of sensitivity to technique, it is difficult to compare results generated by different laboratories.

Turbidimetric test: As clot formation proceeds the reaction mixture becomes increasingly more turbid with the production of insoluble clotting protein (Levin and Bang, 1968). By following the development of turbidity spectrophotometrically it is possible to improve the accuracy and sensitivity of the LAL test (Levin and Bang, 1968; Teller and Kelly, 1979). The turbidimetric assay procedure can be performed as either an endpoint test, where the measurement of optical density is made at the end of a defined incubation period, or as a kinetic test, where the time elapsed from addition of sample to the production of a required change in optical density is measured (Wachtel and Tsuji, 1977). For kinetic tests continuous, or at least periodic, optical density measurement is required.

The greatest source of error in the endpoint test is in mis-timing the optical density measurement at the end of the incubation period (Valois, 1979). The turbidimetric methods have distinct advantages over the gel-clot test in that they remove the subjective decision of what constitutes a firm gel and allows LPS concentrations to be determined over a continuous range. Turbidimetric assays also demonstrate greater sensitivity, precision, and accuracy over the gel-clot test.

Chromogenic test: The previous methods have used all the naturally occurring enzymes of the Limulus coagulation system (Berzofsky and McCullough, 1991). In the chromogenic assay methods the natural LAL coagulogen is replaced with a synthetic substrate which can be modified by the activated coagulase (Sloyer and Karr., 1982). The initial portions of the LAL enzyme cascade are still present (Sloyer and Karr, 1982). The synthetic substrate consists of a 5 amino acid polypeptide (5Pep) which is bound to p-nitroaniline (pNA). The coagulase is able to cleave the 5Pep-pNA bond liberating free pNA which can be measured spectrophotometrically (Sloyer and Karr, 1982; Friberger and Mellstam, 1982). The rate of pNA liberation is a function of the amount of endotoxin in the sample (Wachtel and Tsuji, 1977; Albaugh and Chandler, 1982). The chromogenic assay can be performed as either an endpoint or a kinetic test (Sloyer and Karr, 1982). As in the turbidimetric assay standard curves can be created and used to quantitate endotoxin in unknown samples (Sloyer and Karr, 1982).

Advantages of the chromogenic endotoxin assay when compared to the traditional gel-clot assay include: 1) The test is quantitative over a continuous range; 2) The response is measured objectively; 3) It takes less time to perform; and 4) The test uses less LAL reagent.

The chromogenic test has advantages over the turbidimetric test: 1) It is more sensitive by being able to quantify LPS levels as low as 5 pg/ml and 2) The endpoint method obviates the problem of the reaction proceeding after incubation by stopping the reaction at a set time.

The chromogenic tests show precision equal to or greater than the turbidimetric tests. Endpoint tests typically show RSD's of 2-4% for assays run on the same day and RSD's for assays run on different days.

Recommendations

The following procedure is recommended for the collection, storage, extraction, and analysis of airborne dust samples for endotoxin.

1. Sample Collection: Personal samplers using glass fiber filters designed to collect either total dust or the thoracic fraction.

2. Sampling duration is dependent on the dust level and objective of the study. For most environments sampling time is not critical.
3. Sample extraction: Pyrogen free water with gentle shaking for 60 minutes followed by immediate analysis of the samples. Centrifugation may be necessary for samples containing particulate matter.
4. Extract storage: If samples must be stored for more than 24 hours, they should be frozen at -80 C in either polystyrene or borosilicate glass containers. If samples can be analyzed within 24 hours of extraction they should be stored at 4 C prior to analysis.
5. Until a standardized procedure is developed it is recommended that laboratories with experience in measuring environmental endotoxin with the LAL assay be used to evaluate airborne samples; and that these laboratories report results, using appropriate control standard endotoxin, in units of EU's per unit weight or volume.

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