COMPARISON OF *LIMULUS* AMEBOCYTE LYSATE TEST AND GAS CHROMATOGRAPHY MASS SPECTROMETRY FOR MEASURING LEVELS OF ENDOTOXIN

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Abstract

A study of the precision of LAL and GC-MS analyses for endotoxin measurement was conducted by comparing the levels of endotoxin in pure LPS (*Escherichia coli* serotype 055:B5) solutions. The objectives of the study were to establish and validate the GC-MS technique analyzing specific 3-hydroxy fatty acids (3-OH 14:0 and 3-OH 16:0) as chemical markers of endotoxins, and to evaluate the correlation between the GC-MS technique and the LAL technique using samples of pure LPS (*E. coli*), pure Gram-negative bacteria (*E. agglomerans*), and a cotton dust which contains different types of Gram-negative bacteria and physical states of endotoxin. Calibration curves for the 3-OH 14:0 and 3-OH 16:0 (with 3-OH 13:0 as the internal standard) were constructed with concentrations injected into the GC-MS ranging from 2 to 46 ng (r^2=0.9993 in both cases). Sample recovery of the 3-OH 13:0, 3-OH 14:0, and 3-OH 16:0 was determined to be an average of 80%, 82%, and 82% respectively. The ratios (GC-MS:LAL) of the levels of endotoxin found in the *E. coli*, *E. agglomerans*, and cotton dust samples were 2:1, 20:1, and 10:1, respectively. The limit of detection (LOD) of the GC-MS analyses was an average of 0.300 ng/ul which was approximately one thousand times greater than the average LOD of the LAL assays.

Introduction

Studies have shown that various occupational environments contain airborne organic dusts which may cause significant health effects upon exposure (Reynolds and Milton, 1993). One constituent of these airborne dusts that may be associated with health effects is endotoxin. Endotoxins are a major component of the outer membrane of Gram-negative bacteria, and are composed of complex lipopolysaccharides (LPS). The LPS is comprised of three parts: the O-specific polysaccharide chains, a core polysaccharide, and a lipid portion (lipid A) (Maitra et. al., 1986). The lipid A backbone of the LPS contains four moles of beta-hydroxy fatty acids which have been used as chemical markers for endotoxins during the analysis of Gram-negative bacteria (Maitra et. al., 1986). The majority of the toxicity associated with endotoxins is contained within the lipid A portion of the LPS (Rylander and Jacobs, 1994).

There are several types of analyses which may be used to detect and quantify endotoxin; however, for airborne organic dust the most common test is the *Limulus* Amebocyte Lysate (LAL) assay. This analysis is highly sensitive to endotoxins. The LAL assay measures the level of biologically active endotoxins within a sample (Saraf and Larsson, 1996). Although this method of analysis is highly sensitive and reliable, there are common environmental contaminants which may interfere with the assay (Jacobs, 1989). For this reason alternative methods are being considered to evaluate environmental endotoxins. A second type of analysis used to measure endotoxin is gas chromatography-mass spectrometry (GC-MS). GC-MS can measure substances which are unique to microorganisms, such as fatty acids or peptidoglycan. These unique chemical markers have been used to estimate the numbers of microorganisms and levels of specific microbial products that are present in the environmental samples. The chemical markers used for determining endotoxin are 3-hydroxy fatty acids (or beta-hydroxy fatty acids) (Saraf and Larsson, 1996). Since the GC-MS analysis determines the amount of these specific chemical markers, the interferences with the endotoxin samples analyzed using the LAL assay may be avoided. This study set up the GC-MS assay for endotoxin as described by Mielniczuk, et al. (Mielniczuk et. al., 1993; Mielniczuk et. al., 1992), and then evaluated the agreement between the GC-MS assay and the LAL assay for pure LPS, a Gram-negative bacteria preparation, and cotton dust.

Materials and Methods

LAL Assay for Endotoxin

Dilutions of the sample extracts were assayed using the quantitative kinetic chromogenic LAL analysis method (Kinetic-QCL; BioWhittaker), a BioWhittaker microtiter plate reader, and individually packaged sterile microtiter plates (Corning, Inc.--Corning, New York). The endotoxin concentration was determined by measuring the onset time, the time required for the sample to reach a 200 mOD increase in optical density, after addition of the lysate. Dilutions of the control standard endotoxin (CSE), available with the LAL assay kit, were used to construct a standard curve for determining the concentration of endotoxin in the samples. Data were expressed as Endotoxin Units (EU).

GC-MS Analysis

Chemicals: Tridecanoic, tetradecanoic, and hexadecanoic acids hydroxylated in position 3 (3-OH 13:0, 3-OH14:0, and 3-OH 16:0, respectively) were used to prepare standards for the assay. Bis(trimethylsilyl) trifluoroacetamide (BSTFA, 98%), methanol, acetyl chloride, n-hexane (99%), helium, dichloromethane, hydrochloric acid, diethyl ether, nitrogen and pyridine were also used during the analysis. All glassware was heated overnight at 350°C in order to be

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pyrogen free before use (Mielniczuk et. al., 1993; Mielniczuk et. al., 1992).

Derivatization of Hydroxy Acids/Construction of Calibration Curves: Stock solutions of tridecanoic, tetradecanoic, and hexadecanoic acids were prepared by dissolving 1.25 mg of each acid (tridecanoic, tetradecanoic, and hexadecanoic) into 5 ml of hexane-diethyl ether (4:1, v/v). Due to the instability of 3-OH fatty acids, the tridecanoic used as an internal standard was esterified before storage. The methyl esters of the free hydroxy acids 3-OH 14:0, 3-OH 16:0, and 3-OH 13:0 were prepared by heating 250 µg of each acid in 1 ml of 1.3 M methanolic HCl at 80°C for 30 minutes in a heating block. Methanolic HCl (1.3 and 3.6 M) was prepared by adding acetyl chloride (1 or 3 ml) dropwise into methanol (9 or 7.5 ml) at 0°C. One ml of distilled water was then added each fatty acid preparation and the samples extracted twice with 1.5 ml of n-hexane. The combined hexane phases were evaporated to dryness under a stream of nitrogen. The sample was redissolved in 1 ml of n-hexane, divided into five equal parts (containing 50 µg of each acid), and evaporated to dryness under a stream of nitrogen. Each of the samples were then subject to TMS derivatization (Mielniczuk et. al., 1993; Mielniczuk et. al., 1992). The TMS derivatization was done by adding 50 µl of BSTFA and 5 µl of pyridine to the sample. The sample was then heated at 80°C for 15 minutes in a heating block. To construct calibration curves, one microliter of the sample was injected into the GC-MS and analyzed with the GC-MS at the conditions described below (Mielniczuk et. al., 1993; Mielniczuk et. al., 1992).

Preparation of Samples for LAL and GC-MS Analysis

LPS for GC-MS Analysis: LPS samples were prepared by suspending 0.5 mg of *E. Coli* 055:B5 LPS in 4 ml of PFW and diluting to a concentration range on a weight basis of 0.2 to 25 ug/ml (at concentrations of 25, 5, 1, and 0.2 ug/ml). The first working solution of 25 ug/ml was made by adding 0.4 ml of stock solution and PFW for a total volume of 2 ml. The second, third, fourth, and fifth working solutions were made in this manner with the addition of 0.4 ml of the previous working solution to PFW for a total volume of 2 ml. An aliquot of each dilution was evaluated by the LAL assay and the results were expressed as EU/ng relative to the control standard endotoxin (CSE) and converted to ng/ml using a conversion factor of 10 EU/ng.

For GC-MS analysis, one milliliter of each dilution was transferred to test tubes with Teflon-lined screw caps. These samples were heated in a heating block at 100°C for 18 hours in 3.6 M methanolic HCl. The methyl esters were then extracted with n-hexane and distilled water as previously described. The hexane layer was evaporated using a stream of nitrogen, and the sample was redissolved in 1 ml of hexane-dichloromethane (1:1, v/v). One milliliter of the sample was applied to a silica gel column to separate the methyl esters of hydroxylated acids from non-hydroxylated acids. The column was washed with 1 ml of hexane-dichloromethane before use. After the sample was applied to the column, 0.6 ml of hexane-dichloromethane was added. The hydroxy fatty acid methyl esters were eluted with 1.5 ml of diethyl ether, and the solvent was evaporated with a stream of nitrogen. The samples were combined with 1.25 ug of 3-OH 13:0, and the TMS derivatization was performed as described above. The sample was analyzed by injecting 1 µl into the GC-MS in the EI (electron-impact) mode (Mielniczuk et. al., 1993; Mielniczuk et. al., 1992).

E. agglomerans: The sample was prepared by suspending 5 mg of *E. agglomerans* (50:50 whole cell:disrupted cell prepared by Ribi ImmunoChem Company in Hamilton, Montana) in 4 ml of PFW. The supernatant was divided so that the samples contained levels of *E. agglomerans* in the range of 2 to 250 ug/ml of *E. agglomerans* on a weight basis (at concentrations of 250, 50, 10, and 2 ug/ml). Dilutions of the *E. agglomerans* solution were prepared as previously described for *E. coli* LPS. Aliquots of each solution were prepared and analyzed by the LAL and GC-MS as described above.

Cotton Dust: Two hundred milligrams of cotton dust were placed into a sterile centrifuge tube with 4 ml of PFW and shaken for one hour at room temperature. The extract was centrifuged at 1,000 x g for 10 minutes at room temperature. The supernatant was aliquoted so that the samples contain levels of dust in the range of 1 to 20 mg/ml on a weight basis (at concentrations of 20, 8, 3.2, and 1.28 mg/ml). The first working solution of 2000 ng/ml was made by adding 0.8 ml of stock solution and PFW for a total volume of 2 ml. The second, third, fourth, and fifth working solutions were made in this manner with the addition of 0.8 ml of the previous working solution to PFW for a total volume of 2 ml. Aliquots of each solution were prepared and analyzed by the LAL assay and GC-MS as described above (Mielniczuk et. al., 1993; Mielniczuk et. al., 1992).

Gas Chromatography - Mass Spectrometry

The GC-MS used was a Hewlett-Packard 5890 MSD equipped with a HP-5 fused-silica capillary column (30M x 0.25mm x 0.25µm). Injections of the sample were made with a syringe into the GC in the splitless mode. The helium carrier gas had a flowrate of 2 ml/min through the column, at an inlet pressure of 7 psi. The temperature of the column was programmed at 120 to 260°C at 20°C/min. The injector and the interface between the GC and MS remained at 260°C. TMS derivatives were analyzed in the EI mode with an ion source temperature of 220°C (Mielniczuk et. al., 1993; Mielniczuk et. al., 1992).

Data Analysis

The amount of endotoxin in a sample analyzed by GC-MS was determined by individually comparing the peak areas of both the 3-OH 14:0 and 3-OH 16:0 with the peak area of the internal standard (3-OH 13:0). This comparison allowed the calculation of the unknown mass of the 3-OH 14:0 and 3-OH 16:0 from the known mass of the internal standard. From these data, the number of moles of 3-OH fatty acids in LPS

can be calculated. Theoretically there are four moles of fatty acid per LPS molecule (Walters et al, 1994), therefore the total number of moles of fatty acid was divided by four to determine the number of moles of LPS present in the sample. The number of moles of LPS was then multiplied by 8000, the average molecular weight of LPS (Walters et al, 1994), in order to determine the mass of LPS per milliliter of solution. Using this procedure the endotoxin levels (ng/ml) were calculated for the stock solution and the four dilutions of each of the samples--*E. coli*, *E. agglomerans*, and cotton dust. To compare the levels measured by GC-MS with those measured by LAL, regression analysis was performed in which the GC-MS data were considered the independent variable and the results from the LAL assay as the dependent variable. The correlation coefficient (r^2) was calculated for each comparison using Quattro Pro statistical software.

Results And Discussion

Using GC-MS analysis calibration curves for the 3-OH 14:0 and 3-OH 16:0 (with 3-OH 13:0 as the internal standard) were constructed with injected concentrations ranging from 2 to 46 ng/µl. The correlation coefficient indicated good linearity over this range of concentrations (r^2=0.9993). These calibration curves were constructed by direct injection of TMS derivatized fatty acids in the GC-MS.

To determine if the procedure used to prepare environmental samples for GC-MS analysis (overnight heating of the samples in methanolic HCl prior to TMS derivatization) affected the recovery of the fatty acids, a portion of the fatty acids were analyzed as standards, and the remaining samples were analyzed as environmental samples. The average recoveries of the environmentally treated 3-OH 13:0, 14:0, and 16:0 fatty acids were 80%, 82%, and 82%, respectively. This suggests that the total endotoxin concentration may be under estimated by 20% due to the loss of fatty acid mass during sample preparation. Further studies are needed to confirm this observation. For the purposes of this study, no correction was made to account for this loss in calculating the endotoxin levels for the GC-MS environmental samples.

The correlations between the GC-MS and LAL methods for the *E. coli*, *E. agglomerans*, and cotton dust samples were 0.996, 0.972, and 0.862, respectively (Figures 1, 2, and 3). These data indicate a high correlation between the results from the GC-MS and the LAL test for the three types of samples. However, the amount of endotoxin determined by the GC-MS method was much higher than that determined by LAL assay. This is also shown in Table 1 which compared the concentration of the stock solutions of each of the three samples by GC-MS with the LAL results. For *E. coli*, the levels estimated by GC-MS were twice as high as those estimated by the LAL, and the differences were even greater for the cotton dust (10 fold) and *E. agglomerans* (20 fold). The higher levels measured by the GC-MS were expected, because the technique is thought to quantify all of the 3-OH fatty acids in a sample. This is because it is a chemical assay designed to determine the total amount of a specific chemical constituent in a sample. Therefore prior to derivatization, a rigorous extraction technique is used to liberate all of the 3-OH fatty acids. However, the LAL assay is only able to quantify the amount of endotoxin which is extracted in PFW and which can biologically activate the assay's enzyme cascade.

For the purified *E. coli* LPS, one would expect that the LAL would detect all of the LPS in the sample. The two fold higher concentration detected by GC-MS is likely to have resulted from the limited solubility of the pure LPS in PFW used to extract for the LAL analysis. For the *E. agglomerans*, the 20 fold difference may be related to the fact that the preparation consisted of whole cells and cell wall fragments that would not be as efficiently extracted by PFW for the LAL analysis.

For cotton dust, differences in the extraction efficiency may not adequately explain the differences in measured levels. This is because the diverging lines in Figure 4, which compares the individual response curves for the LAL and GC-MS and cotton dust, suggest that at the higher concentrations of the cotton dust there is an interference with the LAL assay. Such interferences are frequently reported for the LAL assay and the lower LAL results for this study may have been caused by interfering components in the cotton dust.

In this study, the GC-MS was more reliable (as it appears to have no interferences) but not more sensitive than the LAL assay. The average limit of detection (LOD) for the *E. coli*, *E. agglomerans*, and cotton dust samples was 0.300 ng/µl which was calculated using the formula: LOD=3(variance of the intercept)$^{0.5}$/slope); whereas, the average LOD for the samples analyzed by LAL assay in this study was approximately one thousand times less than that of the samples analyzed by GC-MS. The LOD for the samples analyzed by GC-MS in the study by Mielniczuk, et al (Mielniczuk et. al., 1993; Mielniczuk et. al., 1992) was approximately one hundred times less than the LOD for the samples analyzed by GC-MS in this study. This difference is most likely caused by the greater sensitivity of the GC-MS used by Mielniczuk et al (Mielniczuk et. al., 1993; Mielniczuk et. al., 1992) . Nonetheless, the sensitivity of the GC-MS used in the Mielniczuk et al (Mielniczuk et. al., 1993; Mielniczuk et. al., 1992) study was still at least ten times less than that of the LAL assay used in this study. Since the LOD for the GC-MS is much greater than that of the LAL assay, when performing air monitoring surveys it would be necessary to collect much more sample if GC-MS, rather than LAL assay, is the chosen method of analysis.

Conclusions

1. The results of the GC-MS analyses and LAL tests for the *E. coli*, *E. agglomerans*, and cotton dust were highly

correlated. The highest correlation was for *E. coli* which may be related to it being a purified LPS preparation.

2. Although the results of the GC-MS and the LAL analyses were highly correlated, the levels measured by GC-MS analyses were consistently higher than those of the LAL analyses for all samples. These differences are accounted for in part by the differences in the extraction efficiencies of the two techniques and by the fact that the LAL measures only the endotoxin that can biologically activate the assay's enzyme cascade.

3. For the cotton dust sample, the differences in measured levels may also be related to the presence of substances that interfere with the LAL assay. Substances that interfere with the LAL assay are likely to be common components of organic dust and represent a major problem in comparing the results of endotoxin measurements by LAL with those by the GC-MS technique.

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Table 1. Calculated Concentrations of Endotoxin Contained in Various Stock Solutions Based on the Results of GC-MS and LAL Analyses of Diluted Samples.

GC-MS		LAL
Substance	LPS (ng/ml)	LPS (ng/ml)
E. coli (125ug/ml solution)	257,552	111,442
E. Agglomerans (1.25mg/ml solution)	9,526	471.82
Cotton Dust (supernatant from 50mg/ml mixture)	61,617	6073.8

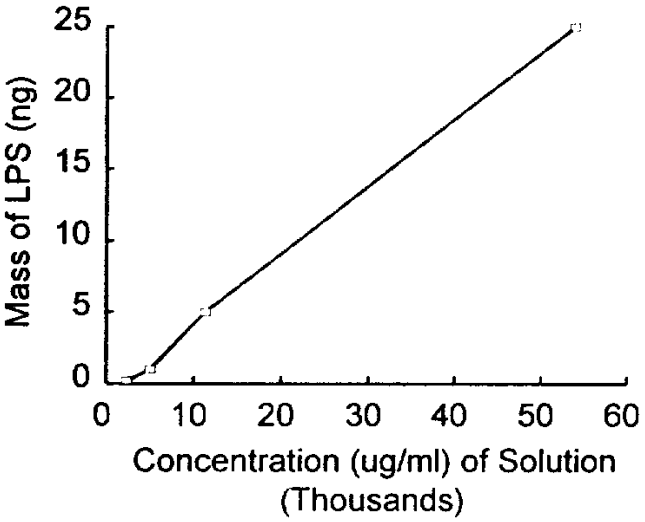

Figure 1. E. coli LPS.

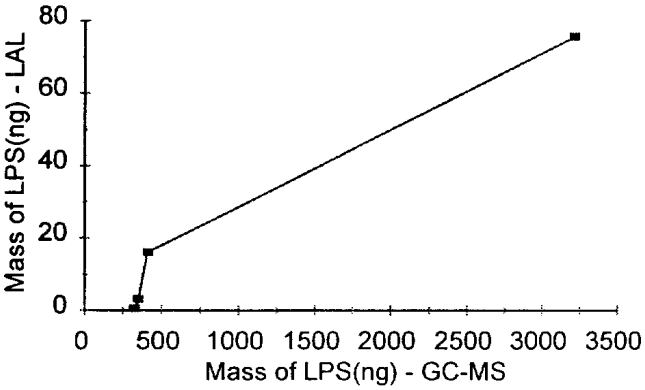

Figure 2. Enterobacter agglomerans.

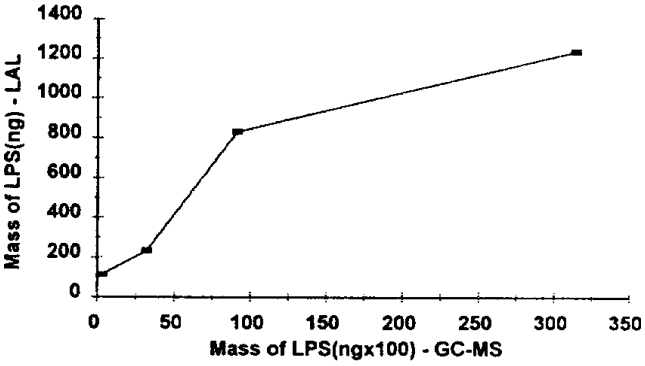

Figure 3. Cotton Dust.

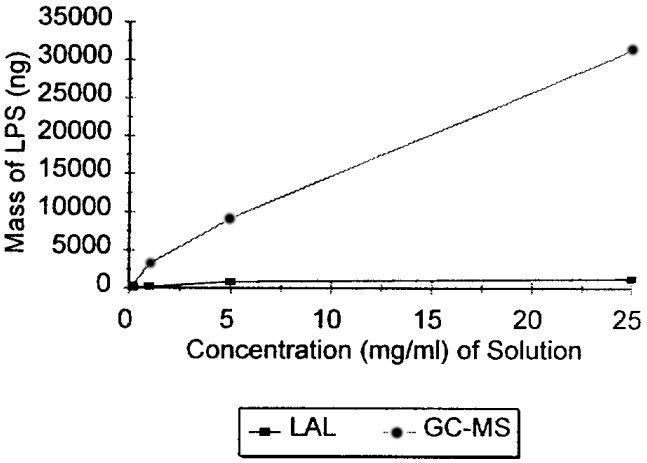

Figure 4. Cotton dust GC-M vs. LAL.