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Determination of lipid A and endotoxin in serum by mass spectroscopy

(β-hydroxymyristic acid/gas chromatography-mass spectrometry/Gram-negative sepsis)

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ABSTRACT **A quantitative technique for determining lipid A content of endotoxin added to serum by combined gas chromatography-mass spectrometry is described. This technique uses detection of the β-hydroxymyristic acid content of *Salmonella minnesota* R595 lipopolysaccharide by selected ion monitoring at atomic mass unit of 315.4 The fatty acids produced on hydrolysis of serum containing lipopolysaccharide were extracted and the methyl esters were made. Silica gel chromatography was used to separate methyl esters of hydroxy fatty acids from other fatty acid methyl esters. Trimethylsilyl ether derivatives of the hydroxy fatty acid methyl ester fraction were quantitated by this technique. As little as 200 fmol of β-hydroxymyristic acid could be detected.**

Gram-negative bacillary sepsis continues to be an important clinical entity with a high mortality rate (1–3). Based primarily on animal studies (1, 3–6), it appears that many of the physiological consequences of gram-negative bacteremia are causally related to the presence of bacterial lipopolysaccharides, namely, endotoxin. Endotoxin is a complex substance located in the cell wall of Gram-negative bacteria and is composed of *o*-specific side chains, a core polysaccharide, and a lipid moiety called lipid A (7–14). Extensive studies by several investigators (9, 10, 15) have suggested that the "toxic" component of endotoxin resides in this lipid-A moiety. Lipid A has a molecular weight of approximately 1700, with its major constituents being D-glucosamine (20–30%), phosphate (2%), and long-chain fatty acids (60–70%) (10, 11, 16). β-Hydroxy [β(OH)] fatty acids comprise the major fatty acids and are unique in that they have not been reported in significant amounts in normal human serum (17). The most commonly occurring β(OH) fatty acid in Gram-negative bacteria is β-hydroxymyristic acid, which is present in lipid A or endotoxin (14). Thus, this unique compound could be useful as a "marker" for the presence of lipid A or endotoxin (18).

Previous attempts at determining endotoxin activity have been dependent on biological assay systems. These include the pyrogen test (19), fever index (20), animal lethality (21), epinephrine skin test (22), and synovial inflammatory response (23). Also, the *Limulus* lysate gelation test has been developed as a means for detecting circulating endotoxin (24). Disappointingly, the clinical correlation with this last assay has been unsatisfactory because of the significant incidence of false-positive and false-negative results (25). Moreover, like the other bioassay systems, the *Limulus* lysate test fails to measure endotoxin directly but rather attempts to quantitate some biological effect. On the basis of the chemical structure of lipid A, we have developed a method to detect endotoxin in serum by quantitating β(OH) myristic acid by means of gas chromatography-mass spectrometry. Mass spectrometry has been used successfully in the past to detect various biological compounds (26). This procedure is highly specific and very sensitive. The method described in this communication quantitates nanogram quantities of lipid A and endotoxin in serum.

MATERIALS AND METHODS

Serum Samples. New Zealand white rabbits weighing approximately 2 kg, and not fed overnight, were used. Blood was obtained by heart puncture under sterile conditions. Blood was also obtained aseptically from human volunteers by venipuncture. Serum was separated by centrifugation and stored in pyrogen-free tubes at −20°.

Solvents. All solvents were either freshly distilled or were liquid chromatography grade and purchased from Burdick and Jackson Co.

Determination of β(OH) Myristic Acid Content of Lipid A. Varying amounts of *Salmonella minnesota* R595-lipid A or *Escherichia coli* 0127:B8 endotoxin in 5 ml of pyrogen-free 0.85% wt/vol NaCl or in 3–5 ml of rabbit or human serum were hydrolyzed with 5 ml of 8 M HCl for 4.5 hr at 110°. The free fatty acids of the hydrolysate were extracted three times with 10 ml of diethyl ether. The ether extracts were pooled, washed with 5 ml of pyrogen-free water, and dried under nitrogen. The extracted free fatty acids were methylated by the procedure of Schlenk and Gellerman (27), dried under nitrogen, dissolved in 0.5 ml of pentane, and applied to a dry silica gel column 60–200 mesh (0.5 × 3 cm). The column was eluted with 3% ether in pentane to remove saturated and unsaturated methyl esters of fatty acids (28). The remaining polar fatty acids, including hydroxy fatty acid methyl esters, were eluted quantitatively with 20% ether in pentane and dried under nitrogen. After addition of 0.25 ml of trimethylsilyl reagent (Me_3Si imidazole, Applied Science), the samples were shaken on a Vortex mixer. After 2 min, 1 ml of water was added. The Me_3Si ethers of hydroxy fatty acid methyl esters were extracted three times with 1 ml of pentane. The pentane extracts were pooled and dried carefully under a nitrogen stream at 30°. The dried Me_3Si ethers were dissolved in 0.1 ml of acetonitrile, mixed on a Vortex, and filtered through pasteur pipettes plugged with silanized glass wool.

Two to five microliters of the acetonitrile solution were injected into a gas chromatograph-mass spectrometer (model 5992 A, Hewlett-Packard, Palo Alto) equipped with a membrane separator. The relative abundance at 315.4 atomic mass unit was determined by using a selected ion monitoring program in conjunction with a six-ion data acquisition program available from Hewlett-Packard. The glass column (2 mm inner diameter × 1.8 m) was packed with 3% Silar-10C on 100/120

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Abbreviations: β(OH), β-hydroxy; Me_3Si, trimethylsilyl; Me_3Si-Me-β(OH)C_{14}, trimethylsilyl ether of β(OH) methyl myristate.

gas chrom Q (Applied Science Laboratories). The column and injection port temperatures were 180° and 240°, respectively). Zero helium (Liquid Carbonic) was used as a carrier gas at a flow rate of 30 ml/min. The electron multiplier voltage setting was 2800.

Fragmentation Pattern of Me_3Si Ether of β(OH) Methyl Ester of Myristic Acid [Me_3Si-Me-β(OH)C_{14}] and Its Pentadeuterated Derivative. A Hewlett-Packard 5992A gas chromatograph-mass spectrometer with a peak finding program was used. Me_3Si-Me-β(OH)C_{14} showed mass fragmentation of high abundance at atomic mass units of 315.4 and 175 (Fig. 1). We have obtained a pentadeuterated β(OH) myristic acid from Merck, Sharp & Dohme, Canada Ltd. Montreal, Canada. The mass fragmentation pattern of this derivative is also shown in Fig. 1. The pattern is essentially identical to the nondeuterated derivative except the major mass peak is at 320.4 rather than at 315.4 and the second major peak is seen at 178.

Standard Curve and Cross Talk. Known concentrations of both the nondeuterated and pentadeuterated Me_3Si-Me-β(OH)C_{14} were prepared and the relative abundance at 315.4 for the nondeuterated and at 320.4 for the pentadeuterated derivative were measured. These standard curves were linear when plotted on double logarithmic graph paper for both derivatives. The range covered in these experiments was 200 fmol–1000 pmol (Fig. 1). At the lower limit, 200 fmol produced a relative abundance of about 350 at *m/e* 315.4. The background under these conditions was less than 50. Cross talk (overlap of 315.4 from 320.4 or vice versa) for both derivatives

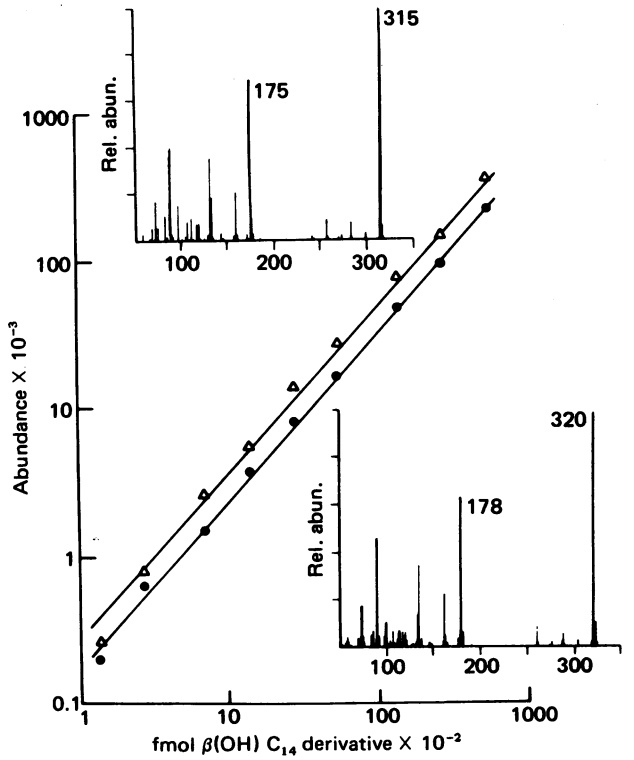


FIG. 1. Relationship of mass abundance of Me_3Si-Me-β(OH)C_{14} to concentration. Me_3Si-Me-β(OH)C_{14} and the pentadeuterated derivative were prepared as described in the text. △ and ●, Nondeuterated (*m/e* 315.4) and pentadeuterated (*m/e* 320.4) derivatives, respectively. Characteristic mass fragmentation peaks at 315 and 175 are observed for the nondeuterated derivative (*Upper Inset*). Similarly, mass peaks at 320 and 178 are noted for the pentadeuterated derivative (*Lower Inset*). For both insets the ordinates represent relative abundance with a full scale of 100. The abscissas represent *m/e*.

at concentrations below 1000 fmol was less than 1% and was not significant at higher concentrations. When an equimolar mixture of both derivatives was diluted from 1:10,000 to 1:1,000,000, the observed ratio (1.415) of nondeuterated to pentadeuterated derivative remained unchanged.

Internal Standard. A pentadeuterated β(OH) myristic acid was used as our internal standard. The five deuterium atoms are located on carbons 2, 3, and 4. Initially, the internal standard was added prior to hydrolysis of serum. However, our experimental data showed that this deuterated standard was partially destroyed during hydrolysis. The kinetics of this reaction will need further study to minimize the loss. In the present study the internal standard was added after hydrolysis. Thus, the values obtained are minimum values for β(OH) myristic acid content of serum.

Extraction and Separation of Serum Lipids. Three milliliters of serum were extracted with chloroform/methanol/water 8:4:3 vol/vol (29). The aqueous extract was removed and washed with chloroform. The organic phase was removed and dried under nitrogen. The lipids were separated by silica gel column chromatography (0.5 × 6 cm), with chloroform, acetone, and methanol as eluting solvents. The eluted lipids and the remaining silica gel were dried and used for further analysis.

Liquid Chromatography. Fatty acid methyl esters were separated with a liquid chromatograph (model ALC/GLC 200; Waters Associates). This instrument was equipped with a model 6000 solvent delivery system, a model U6K Universal Injector, and a Micro-Bondapak C_{18} column (30 cm × 4 mm), all obtained from Waters Associates. The eluent was monitored by refractive index and recorded on an integrating recorder (model HP3380A; Hewlett-Packard).

Extraction of Lipid A. *S. minnesota* R595 was grown in beef extract broth, harvested, washed twice with water, treated sequentially with ethanol, acetone, and ether, and dried under reduced pressure. The glycolipid was extracted by the method of Galanos *et al.* (30) and lipid A was obtained by hydrolysis with 1% acetic acid (31).

RESULTS

Separation of Methyl Esters of β(OH) Fatty Acids. When serum samples were hydrolyzed and the fatty acids extracted, methylated, and injected into a liquid chromatograph, three major peaks were eluted with methanol as solvent (Fig. 2*A*). The first major peak at 5.2 min coincided with the elution time of β(OH) methyl myristate. The same elution profile for serum was observed whether or not the nondeuterated methyl ester was added before application to the silica gel column. Elution with 3% ether did not remove the nondeuterated methyl ester but did remove almost all other peaks seen in the serum extracts. Increasing the ether content of pentane to 20% resulted in the quantitative elution of nondeuterated methyl ester (Fig. 2*B*). In a methylated serum extract to which no nondeuterated methyl ester was added, there appeared to be a small peak in the 20% ether eluate. This peak is probably due to small amounts of hydroxy fatty acids. These data suggest that silica gel chromatography can be used to quantitatively separate the methyl esters of β(OH) fatty acids from most other methyl esters of fatty acids present in serum.

Observed Compared to Theoretical Ratio of Pentadeuterated to Nondeuterated Derivative. In order to determine the amount of nondeuterated derivative present in serum with an internal standard of pentadeuterated derivative it was necessary to correlate the theoretical ratios of pentadeuterated to nondeuterated derivative to that of the observed ratios. The

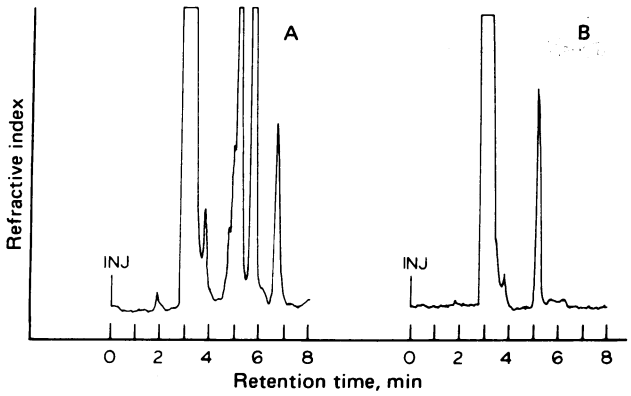


FIG. 2. High-pressure liquid chromatography of fatty acid methyl esters prepared from hydrolysate of rabbit serum. Methanol was used as the eluting solvent at a flow rate of 1 ml/min. Other conditions are described in the text. (*A*) Serum extract plus nondeuterated methyl ester; (*B*) 20% ether eluate of serum extract plus nondeuterated methyl ester.

amount of pentadeuterated derivative added as internal standard was kept constant at 1000 pmol and the amount of nondeuterated derivative was varied. There was a direct correlation of theoretical and observed ratios (Fig. 3). The observed ratio is always less than the theoretical ratio. This is due to the fact that for equimolar amounts of nondeuterated and pentadeuterated derivatives higher abundance is obtained for the nondeuterated derivative (Fig. 1).

β(OH) Myristic Acid Content of Lipid A and Endotoxin. On a logarithm-logarithm graph, a linear relationship was seen between abundance at 315.4 and increasing amounts of lipid A and *E. coli* endotoxin (Fig. 4). The data indicate that the abundance at 315.4 is approximately 10-fold higher for lipid A than for an equal amount of endotoxin.

β(OH) Fatty Acid Content of Normal Human and Rabbit Serum. Three milliliters of serum was hydrolyzed and 1000 pmol of pentadeuterated-Me_3Si-Me-β(OH)C_{14} (20 nmol/ml) were added. The free fatty acids were then extracted and methylated and Me_3Si ether derivatives were prepared. Three peaks at 315.4 atomic mass unit were detected in both the rabbit and human sera (Fig. 5); the first peak eluted (peak I) corresponds to the retention time observed with the pentadeuterated internal standard. Peaks II and III have not been identified. Perhaps they are higher homologs of β(OH) myristic acid.

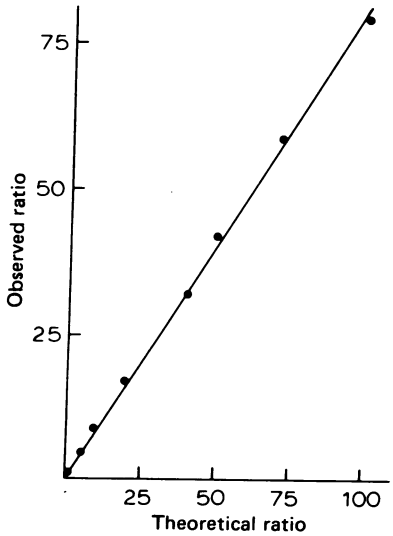


FIG. 3. Observed compared to theoretical ratios of pentadeuterated to nondeuterated Me_3Si-Me-β(OH)C_{14} derivatives. Amount of pentadeuterated derivative was constant (1000 pmol). Nondeuterated derivative was varied from 10 to 1000 pmol. The observed ratio is expressed as the abundances recorded at 320.4 and 315.4.

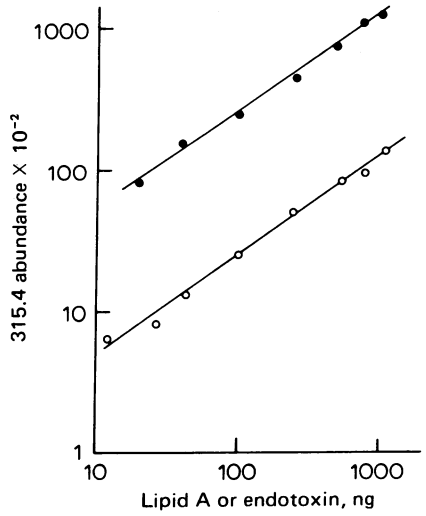


FIG. 4. β(OH) Myristic acid content of lipid A and endotoxin. Various amounts of lipid A and endotoxin in 0.15 M NaCl were hydrolyzed and derivatized. The relative abundance at 315.4 of the Me_3Si ether–methyl ester derivative of β(OH) myristic acid was measured. ●, *S. minnesota* R595 KDO-lipid A; ○, *E. coli* 0127:B8 endotoxin (Difco).

Origin of β(OH) Fatty Acids in Control Serum. As shown in Fig. 5, a significant amount of β(OH) fatty acids was found in normal sera. To determine if these β(OH) fatty acids were derived from circulating endotoxin, lipid A, or other lipids, we separated normal rabbit serum into aqueous and lipid-soluble fractions. It is evident from Fig. 6 that most of the β(OH) fatty acids are lipid soluble. Further fractionation by silica gel chromatography shows that over 80% of the hydroxy fatty acids are eluted with acetone or methanol. This strongly suggests that the β(OH) myristic acids seen on gas chromatography-mass spectroscopy of normal sera are eluted in the acetone and methanol fractions and, therefore, are not derived from endotoxin or lipid A but from other circulating lipids, probably phospholipids. In contrast, lipid A was found to be quantita-

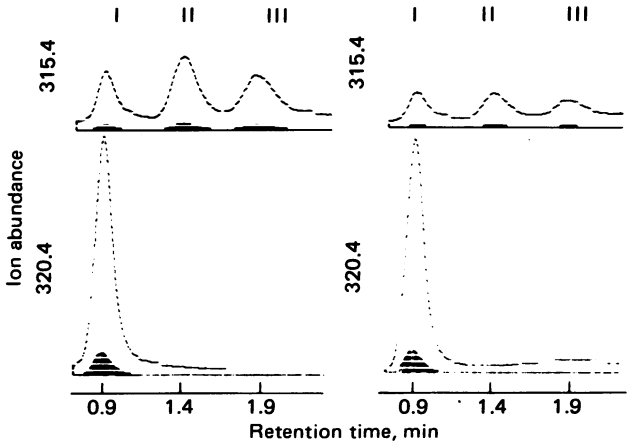


FIG. 5. Computer-assisted selected ion-monitoring scans of normal human (*Left*) and rabbit (*Right*) sera. Sera were hydrolyzed and 1000 pmol of pentadeuterated internal standard were added and derivatized. Mass ions 320.4 and 315.4 were monitored simultaneously. Full scale for human and rabbit sera are 461 and 521, respectively.

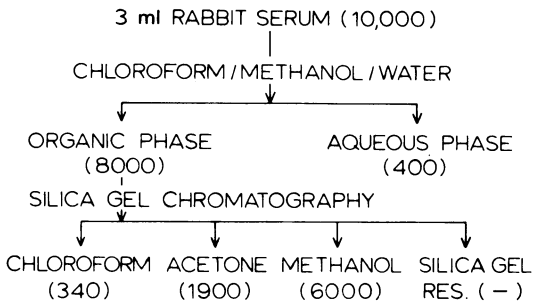


FIG. 6. Partitioning of 315.4 abundance of normal rabbit serum. The values in parentheses are abundance at 315.4 mass unit of peak I (Fig. 5). No internal standard was added.

tively absorbed to silica gel and not eluted with chloroform, acetone, or methanol.

Recovery of Lipid A and Endotoxin Added to Serum. Fifty and 500 ng of KDO-lipid A and endotoxin were added to 5 ml of rabbit serum. The serum was hydrolyzed. After addition of the internal standard, it was extracted and methylated, and Me_3Si ethers were prepared. Table 1 shows that 80–90% of lipid A and endotoxin can be recovered from the serum.

DISCUSSION

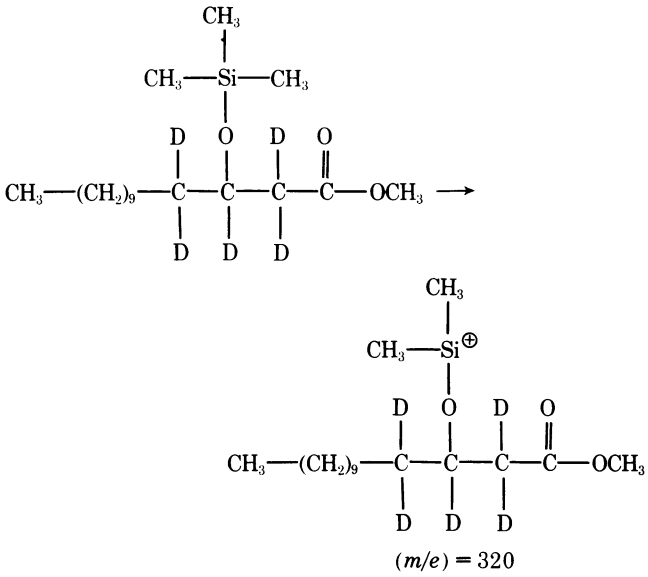
The results described in this investigation indicate the feasibility of measuring circulating levels of lipid A and/or endotoxin. Currently, 50 ng of KDO-lipid A added to 5 ml of serum can be accurately quantitated. This determination is based on hydrolysis of serum followed by analysis of total amount of β(OH) fatty acid. The results presented indicate that normal human and rabbit sera contain approximately 1.5 pmol of β(OH) myristic acid per ml of serum (Fig. 5, peak I). These sera also seem to contain other components detectable at 315.4 (Fig. 5, peaks II and III). Since these components have longer retention times than the β(OH) myristic acid derivitive, it appears that these could be higher homologs of hydroxymyristic acid. The level of total β(OH) fatty acids in normal serum determines, in part, the sensitivity of our procedure. This raises the question as to the source from which the hydroxy fatty acids (315.4 abundance) in normal serum are chemically derived. Separation of normal rabbit serum into lipid-soluble and water-soluble fractions (Fig. 6) indicated that most of the material(s) contributing to 315.4 abundance in normal serum are lipid soluble. On silica gel chromatography, over 80% of these substance(s) giving 315.4 abundance were eluted with acetone and methanol. These data strongly suggest that phospholipids are the compounds primarily responsible for the 315.4 abundance in normal serum. Further, the abundance at 315.4 does not appear to be directly derived from endotoxin or lipid A. Less than 5% of the 315.4 abundance is found in the aqueous phase of chloroform/methanol/water (8/4/3, vol/vol) extract of normal rabbit serum. This could be due to a low level of undetected endotoxemia, possibly associated with bronchial infection with *Bordetella septica*, which is common in rabbits (32). The sensitivity of the procedure described here could be improved by (*i*) removing serum lipids prior to analysis for endotoxin or (*ii*) concentrating the Me_3Si-Me-β(OH)C_{14} derivative to 10 μl rather than to 100 μl (33, 34). These modifications would permit detection of endotoxin at less than 1 ng/ml of serum.

Table 1. Recovery of lipid A and endotoxin added to rabbit serum

Sample	Amount added, ng	Abundance (315.4)	Recovery, %
NaCl + lipid A	50	16,800	
Serum + lipid A	50	40,200	90
NaCl + endotoxin	500	8,000	
Serum + endotoxin	500	32,000	88
Serum		25,000	
NaCl			

S. minnesota R595 KDO-lipid A and *E. coli.* 0127:B8 endotoxin were added to 5 ml of 0.85% NaCl or serum. The samples were hydrolyzed and derivatized. The recovery of lipid A and endotoxin are average values of duplicate runs. Similar values of recovery were calculated from the data shown in Fig. 3.

The characteristic fragmentation of pentadeuterated Me_3Si-Me-β(OH)C_{14} derivative is shown in Fig. 1. The derivation of the 320 base peak is shown below:



Similarly, the nondeuterated derivative gives rise to a base peak at 315. Lipid A isolated from a variety of Gram-negative bacillary organisms has mainly β(OH) myristic acid (14). However, the presence of other β(OH) fatty acids has been reported. It will be important to test whether these β(OH) fatty acids have similar fragmentation patterns.

The large amounts of saturated and unsaturated fatty acids injected in conjunction with the relatively small amount of β(OH) fatty acids results in the shut down of the mass spectrometer detector due to excess source pressure. Hence, complete removal of saturated and unsaturated fatty acid methyl ester by silicic acid chromatography is essential in order to detect small amounts of β(OH) fatty acid methyl ester.

The *Limulus* assay has been used in most studies concerned with quantitating endotoxin. Several groups have reported that patients with septicemia occasionally produce negative tests with this assay. Also, it has been noted that patients without septicemia sometimes have a positive reaction with the *Limulus* assay (25).

Our data indicate the feasibility of chemically measuring lipid A in serum, thus obviating indirect bioassays. It is hoped that the gas chromatography-mass spectrometry procedure will permit more insightful studies of the pathophysiology of endotoxemia.

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