Lipopolysaccharide determination by reversed-phase high-performance liquid chromatography after fluorescence labeling

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Abstract

A sensitive method for the quantitative determination of lipid A, the covalently bound hydrophobic component of lipopolysaccharides (endotoxins), has been developed. The determination of lipid A was based on the quantitative measurement of β-hydroxymyristic acid and β-hydroxylauric acid by reversed-phase HPLC. β-Hydroxy acids were liberated from ester and amide linkages in endotoxins by acid catalyzed methanolysis. The resulting methyl esters were derivatized with 9-anthracene-carboxyl chloride, 9-fluorene-carboxyl chloride and 4-(1-pyrenyl)butyric acid chloride and quantified with a fluorescence detector. The effectiveness of the three derivatizing agents was compared.

As internal standards-β-hydroxytridecanoic acid [β-OH(13:0)] and β-hydroxypentadecanoic acid [β-OH(15:0)] ethyl esters were used. The limits of detection of β-hydroxymyristic acid were 0.5 pg for the 9-anthroyl and 2 pg for the fluorenoyl and 4-(1-pyrenyl)butyroyl ester per sample (signal-to-noise ratio of 3). The detection limits of lipopolysaccharide from a smooth strain (*Escherichia coli* 0111:B4) was 20 pg, while that from two rough strains (*E. coli* Nissle 1917 and *Salmonella typhimurium* SL 1181) was 5 pg per sample after methanolysis and derivatization with 9-anthroyl chloride.

1. Introduction

Endotoxins constitute a part of the outer membrane of the cell wall of Gram-negative bacteria [1]. The lipid A moiety of endotoxins is responsible for the toxic effects of lipopolysaccharides (LPS), like fever, tissue necrosis, endotoxic shock and activation of the complement system [2,3].

A common technique for the determination of endotoxins is the *Limulus* amebocyte lysate (LAL) test [3], in which endotoxins activate a clotting cascade in the amebocyte lysate derived from the crab *Limulus polyphemus*. Endotoxins are measured by determination of the induced gel formation. For quantitative measurements of endotoxins, a chromogenic reaction has been coupled to the clotting cascade [4,5]. However, the selectivity of the LAL test remains questionable considering that some non-endotoxic substances are known to activate the LAL clotting enzymes [6,7].

β-Hydroxy acids, especially β-hydroxymyristic acid [8–10] and β-hydroxylauric acid [11], constitute an essential part of lipid A and have been used therefore as chemical markers for the quantitation of lipid A. These acids have been

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analyzed by gas chromatography–mass spectrometry [8–11].

This study aimed at developing a sensitive, specific and reproducible reversed-phase HPLC method for the determination of lipid A by detection of β-hydroxymyristic acid and β-hydroxylauric acid after derivatization with a fluorophore.

The reaction of acid chlorides with hydroxyl groups is known to be fast and to result in high yields. Hence, fluorescent polycyclic hydrocarbons with an adjacent carboxyl chloride group like 9-anthracene carboxylic chloride have been used on several occasions for sensitive detection of compounds containing hydroxyl groups [12–15].

In this work, three reagents [9-anthroyl chloride, 9-fluorenoyl chloride and 4-(1-pyrenyl)-butyroyl chloride] were tested to determine their suitability for fluorescent labeling and quantitation of β-hydroxymyristic and β-hydroxylauric acid esters as endotoxin markers (Fig. 1).

2. Experimental

2.1. Chemicals and glassware

α-Bromoethyl acetate, decanal, undecanal, dodecanal and tridecanal (technical grade, 90%–97%) were obtained from Aldrich (Steinheim, Germany) and distilled twice in vacuo before
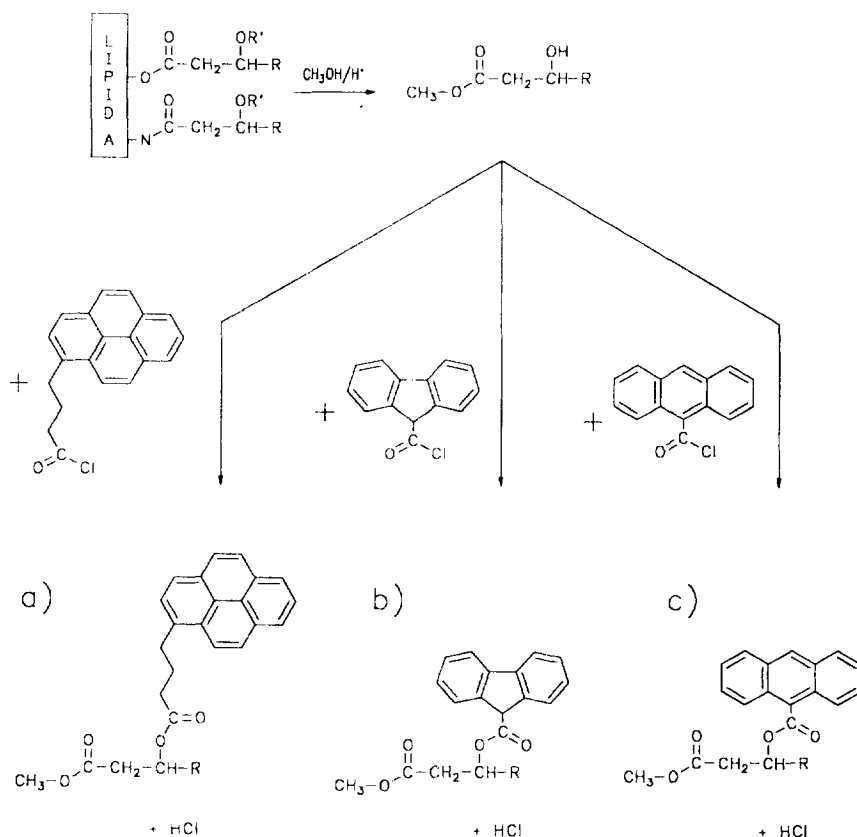

Fig. 1. Formation of β-hydroxy fatty acids from lipid A after methanolysis and derivatization with 4-(1-pyrenyl)butyroyl chloride, 9-fluorenoyl chloride and 9-anthroyl chloride. Resulting products are (a) 3-(4-(1-pyrenyl)butyroyl) fatty acid methyl ester, (b) 3-(9-fluorenoyl) fatty acid methyl ester and (c) 3-(9-anthroyl) fatty acid methyl ester. R:–(CH$_2$)$_{8,10}$–CH$_3$; R':–H or–fatty acid.

use. Zinc powder (99%) and boronic acid tri-methylester (98%, distilled before use) were delivered by Fluka (Buchs, Switzerland). All solvents used for synthesis and purification were analytical grade (Merck, Darmstadt, Germany). 9-Anthracene carboxylic acid, 9-fluorene carboxylic acid and 4-(1-pyrene)butyric acid (Aldrich) were recrystallized three times in acetonitrile before derivatization with thionylchloride (Fluka, distilled twice). Solvents used for chromatography were of chromatography gradient grade (Merck) and used as delivered. Single use solid-phase extraction cartridges, type Lichrolut Si and Lichrolut RP 18 were obtained from Merck. Glassware for analysis was heated to 300°C for 14 h before use. Plasticware was extracted for 6 h in a Soxhlet apparatus with acetonitrile.

2.2. Apparatus and HPLC conditions

^{1}H NMR spectra were recorded with a Bruker WM 250 NMR spectrometer. Excitation and emission spectra were measured with a Shimadzu RF 551 fluorescence spectrophotometer, which was also used for detection during analysis.

The HPLC apparatus was a Merck-Hitachi system with a L-5000 LC low-pressure gradient controller and a C-655A-12 pump. Detector signals were integrated with a Shimadzu CR 3A Chromatopac integrator. Compounds were separated on a reversed-phase column (NuSil 5 μm 120 Å C$_{18}$, 250 × 3 mm I.D., Macherey-Nagel, Düren, Germany) with a guard column (20 × 3 mm I.D.), employing a 20 μl sample loop. Flow-rate was 1 ml/min.

2.3. Synthesis of β-hydroxy acid ethyl esters

β-Hydroxymyristic acid ethyl ester and β-hydroxylauric acid ethyl ester were synthesized for use as reference substances, while β-hydroxy-tridecanoic acid ethyl ester and β-hydroxy-pentadecanoic acid ethyl ester were synthesized as internal standards. Synthesis was performed by Reformatzky reaction from α-bromoethyl acetate and a 10 g mass of the corresponding aldehyde in tetrahydrofuran in the presence of boronic acid trimethylester and zinc powder [16]. The products were extracted with diethyl ether and the diethyl ether solution was washed three times with water. After removal of the organic solvent, the crude product was distilled twice in vacuo. Table 1 shows the yield, the boiling point at 20 mbar, and the molecular mass obtained from mass spectrometry of the product for each aldehyde reacted. Chemical structures of products were verified by ^{1}H NMR.

^{1}H NMR specifications in CDCl$_3$, δ (ppm) were: 0.88 (6H, t,–CH_3), 4.17 (2H, q, CH$_3$–CH_2–CO$_2$–), 4.00 (1H, m,–CHOH–), 2.34 and 2.55 (2H, m, RCO$_2$–CH_2–CHOH–), 1.26–1.38 (16H/18H/20H/22H – (CH$_2$)$_8$– / –(CH$_2$)$_9$– / –(CH$_2$)$_{10}$–/–(CH$_2$)$_{11}$–).

2.4. Synthesis of carboxylic acid chlorides

For fluorescence labeling, carboxylic acid chlorides were synthesized by derivatization of 9-anthracene carboxylic acid, 9-fluorene carboxylic acid and 4-(1-pyrene)butyric acid with thionylchloride in dried toluene [11]. After removal of the solvent, the product was recrystallized three

Table 1
Yields, boiling points and molecular masses determined by mass spectrometry (m/z M$^+$) of β-hydroxy acids (first row) formed by the Reformatzky reaction of an aldehyde (second row) with α-bromoacetic acid ethyl ester

Product (~ethyl ester)	Aldehyde	Yield (%)	b.p. at 20 mbar (°C)	M_r
β-OH(12:0)	Decanal	69	123	244
β-OH(13:0)	Undecanal	74	133	258
β-OH(14:0)	Dodecanal	78	143	272
β-OH(15:0)	Tridecanal	77	152	286

times in dried hexane. During the recrystallization, the humidity of ambient air reacted with acyl chlorides and caused precipitation of insoluble carboxylic acid. These precipitates were removed by filtration of the hot solution through a dried ceramic filter. For color, yield and melting point (uncorrected) see Table 2.

2.5. Synthesis of fluorescence-derivatized β-hydroxy ethyl esters

For determination of fluorescence sensitivity, 1.00 g of β-hydroxymyristic acid ethyl ester, dissolved in 20 ml of dried acetonitrile, was allowed to react with 1.06 g 9-anthroyl chloride, 1.01 g 9-fluorenoyl chloride and 1.35 g 4-(1-pyrenyl)butyroyl chloride, each dissolved in 30 ml of dried acetonitrile (30 min, 22°C). The resulting products were recrystallized three times from ethanol–water. Melting points were determined for 3-(9-anthroyl)myristic acid ethyl ester (white powder, m.p. 37–38°C) and 3-(9-fluorenoyl)myristic acid ethyl ester (white powder, m.p. 36°C). The 3-(4-(1-pyrenyl)butyroyl)myristic acid ethyl ester was a honey-consistent, bright green fluorescent product.

^{1}H NMR specifications in CDCl$_3$ δ (ppm) were:
(1) 3-(9-anthroyl)myristic acid ethyl ester: 0.88 (6H, t,–CH_3), 1.22–1.38 (18H–(CH_2)$_9$–), 1.89 (2H, m,–CHCO$_2$Ar–CH_2–CH$_2$–), 2.73 and 2.90 (2H, m, RCO$_2$–CH_2–CHCO$_2$Ar–), 4.20 (2H, q, CH$_3$–CH_2–CO$_2$–), 5.86 (1H, m,–CHCO$_2$Ar–), 7.50 (4H, m, Ar–H), 8.03 (4H, m, Ar–H), 8.50 (1H, s, Ar–H).
(2) 3-(9-fluorenoyl)myristic acid ethyl ester: 0.88

(6H, t, CH_3), 1.22–1.38 (18H–(CH_2)$_9$–), 1.61 (2H, m,–CHCO$_2$Ar–CH_2–CH$_2$–), 2.51 and 2.63 (2H, m, RCO$_2$–CH_2–CHCO$_2$Ar–), 4.07 (2H, q, CH$_3$–CH_2–CO$_2$–), 4.85 (1H, s, Ar–CH–Ar), 5.34 (1H, m,–CHCO$_2$Ar–), 7.38 (4H, m, Ar–H), 7.65 (2H, m, Ar–H), 7.77 (2H, m, Ar–H).
(3) 3-(4-(1-pyrenyl)butyroyl)myristic acid ethyl ester: 0.88 (6H, t, CH_3–), 1.22–1.38 (20H–(CH_2)$_9$–), 1.61 (2H, m,–CHCO$_2$Ar–CH_2–CH$_2$–), 2.17 (2H, m,–CHCO$_2$–CH_2–CH_2–CH$_2$–CH$_2$–Ar), 2.43 (2H, t,–CHCO$_2$–CH_2–CH$_2$–CH$_2$–CH$_2$–Ar), 2.57 (2H, m, RCO$_2$–CH_2–CHCO$_2$Ar–), 3.37 (2H, t, CHCO$_2$–CH$_2$–CH$_2$–CH$_2$–CH_2–Ar), 4.10 (2H, q, CH$_3$–CH_2–CO$_2$–), 5.29 (1H, m,–CHCO$_2$Ar–), 7.85 (1H, d, Ar–H), 7.94–8.17 (7H, m, Ar–H), 8.29 (1H, d, Ar–H).

The maximum excitation and emission wavelengths, fluorescence intensities and detection limits at individual wavelengths of each compound have been determined after HPLC separation.

2.6. Endotoxin standards

Pure endotoxins were obtained by extracting dried bacteria mass (E. coli Nissle 1917; Ardeypharm, Herdecke, Germany), using the phenol–water extraction procedure [17]. Further endotoxin standards from E. coli serotype 0111:B4 and Salmonella typhimurium serotype SL 1181 (Re mutant) were obtained from Sigma (Deisenhofen, Germany).

2.7. Methanolysis of endotoxins and isolation of β-hydroxy acid methyl esters

Endotoxin from E. coli Nissle 1917 and E. coli 0111:B4 was dissolved in aqueous 0.05% triethylamine solution. The stock solution (1 mg/ml endotoxin) was diluted to give 1 μg/ml endotoxin. The kinetics of methanolysis was investigated using hydrochloric acid (1 mol/l and 4 mol/l in methanol) and sulfuric acid (0.5 mol/l and 2 mol/l in methanol). The acid–methanol mixture was added to 100 μl of the endotoxin

Table 2
Color, yield and melting points of polycyclic hydrocarbon acyl chlorides used for derivatization of β-hydroxy acids

Acid chloride	Color	Yield (%)	m.p. (°C)
9-Anthroyl-	Bright yellow	82	95
9-Fluorenoyl-	Pale brown	85	72
4-(1-pyrenyl)-butyroyl-	Brown	79	82

solution to give a final volume of 5 ml. Temperature during methanolysis was kept at 110°C [9,18–20]. The liberation of β-hydroxymyristic acid as methyl ester from endotoxin was determined as a function of acid concentration and duration of heating (procedure described later).

After cooling, 2 ml of water and 1.5 ml of n-hexane were added to the clear reaction mixture. This was shaken vigorously (2 min) and the resulting emulsion was centrifuged (10 min, 1500 g). The upper hexanoic layer was transferred to a Lichrolut Si cartridge. Washing (4 ml) and elution (double portion of 700 μl) of β-hydroxy acid esters were optimized with various concentrations of tert.-butyl methyl ether in n-hexane. The eluted solution was collected in screwcap vials (vial volume 1.5 ml) and the solvent was removed with a dry nitrogen stream at room temperature.

2.8. Derivatisation of β-hydroxy acid methyl esters

A 100-μl volume of 9-anthroyl chloride, 9-fluorenoyl chloride, or 4-(1-pyrenyl)-butyroyl chloride in acetonitrile (5 mg/ml) was added separately to residues obtained from the above described procedure. The vials were closed with Teflon-sealed screw-caps and allowed to react after vigorous shaking. According to the reported kinetics of acylation of hydroxyl groups with 9-anthroyl chloride [12,15], β-hydroxy acid methyl esters were allowed to react with 9-anthroyl chloride for 20 min at room temperature or kept for 10 min at 60°C.

The reaction mixture could not be employed directly for analysis in HPLC because of the high ratio of reagent to product. At a β-hydroxymyristic acid [β-OH(14:0)] amount of 10 pg, the mass ratio of 9-anthroyl chloride to β-OH(14:0) was 50 millions to one. The initial peak (9-anthracene carboxylic acid) intensity descended too slowly to enable quantitation of the β-OH(14:0) derivative. Therefore, the reaction mixture was adsorbed on a Lichrolut RP 18 cartridge, and excess reagent was removed by an acetonitrile–water mixture.

Before adsorption, C_{18} cartridges were conditioned with 1 ml acetonitrile–water (60:40, v/v) avoiding dry-running of the cartridge filling prior to the addition of the sample. Water (40 μl) was added to the reaction mixture. After 5 min, the solution was applied to a RP 18 cartridge and allowed to adsorb (5 min). The cartridge was washed with 5 ml acetonitrile–water (60:40) and dried by the vacuum-induced air flow (10 min). The fluorescence-derivatized compounds were extracted with acetonitrile. The solvent was removed from eluent with dry nitrogen at room temperature, and the residue was redissolved in 150 μl acetonitrile. This solution was employed directly for HPLC quantitation.

According to the described procedure, β-hydroxylauric acid methyl ester, β-hydroxytridecanoic acid methyl ester, β-hydroxymyristic acid methyl ester, β-hydroxypentadecanoic acid methyl ester and the ethyl esters of the mentioned β-hydroxy acids were esterified with 9-anthroyl chloride, 9-fluorenoyl chloride, and 4-(1-pyrenyl)butyroyl chloride. Fluorescence spectra of the synthesized 24 reaction products were compared with regard to their excitation and emission wavelengths, their signal intensities and their detection limits.

3. Results

3.1. Fluorescence spectra, linearity of standard curve, HPLC separation and detection limits of fluorescence-labeled β-hydroxy acid esters

The synthesized fluorescent products were dissolved in acetonitrile–water (95:5, v/v, 10 ng/ml). From this solution, fluorescence spectra of the anthroyl-, the fluorenoyl- and the 4-(1-pyrenyl)butyroyl)derivatized β-hydroxy acid ethyl esters were recorded (Fig. 2) and the excitation and emission maxima were determined (Table 3). The highest signal intensities were obtained using the following excitation/emission wavelengths: anthroyl derivative: 250 nm/462 nm, fluorenoyl derivative 262 nm/310 nm and 4-(1-pyrenyl)butyroyl derivative: 238
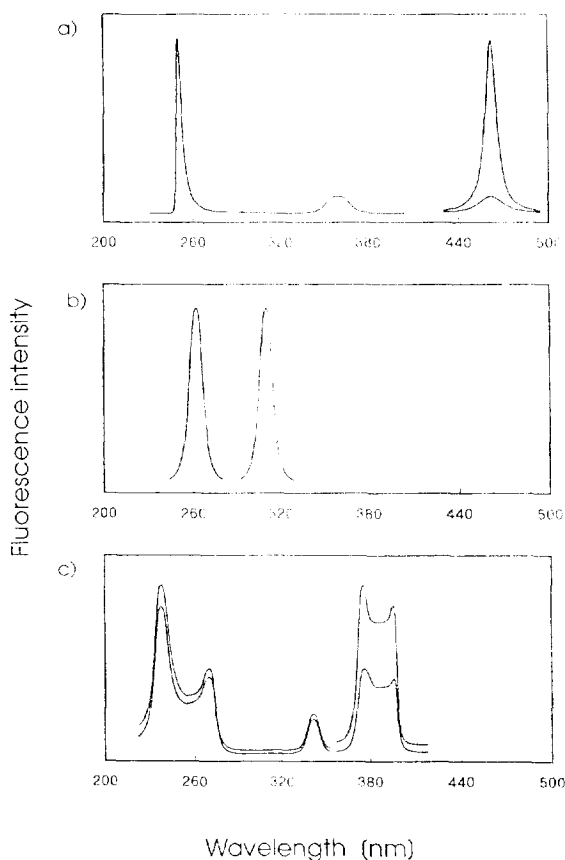
Fig. 2. (a) Fluorescence spectrum of 3-(9-anthroyl)myristic acid methyl ester dissolved in acetonitrile–water (95:5, v/v, 10 ng/ml); excitation wavelength maxima were at 250 nm and 360 nm, emission maxima were at 462 nm for both excitation wavelengths; fluorescence intensity ratio of 462 nm emission at 250 nm excitation to that at 360 nm was 12:1. (b) Fluorescence spectrum of 3-(9-fluorenoyl)myristic acid methyl ester dissolved in acetonitrile–water (95:5, v/v, 10 ng/ml); excitation wavelength maximum was at 262 nm; emission maximum was at 310 nm. (c) Fluorescence spectrum of 3-(4-(1-pyrenyl)butyroyl)myristic acid methyl ester dissolved in acetonitrile–water (95:5, v/v, 10 ng/ml); excitation wavelength maxima were at 238 nm, 270 nm and 336 nm; emission maxima were at 376 nm and 395 nm; fluorescence intensity ratio of emissions (376 nm and 395 nm) at 270 nm excitation to emission at 270 nm excitation was 2.4:1, fluorescence intensity ratio of emission at 376 nm to emission at 395 nm at maximum intensity excitation wavelengths (238 nm and 270 nm) was 1.15:1.

nm/376 nm. These wavelength pairings were used for comparison of sensitivity of the single compounds.

Table 3

Excitation and emission wavelengths of β-hydroxymyristic acid ethyl ester after derivatization with three different polycyclic acyl chlorides

β-Hydroxy acid ethyl ester derivative	Excitation (nm)	Emission (nm)
9-Anthroyl-	**250**, 360	462
9-Fluorenoyl-	262	310
4-(1-pyrenyl)- butyroyl-	**238**, 270, 342	**376**, 396

In case of more than one excitation/emission maxima, the values of the most intensive wavelength combination are in bold.

No influence of the chain length of the β-hydroxy acid esters or the kind of ester (methyl or ethyl ester, spectra recorded prior and after methanolysis) on the fluorescence spectra was observed. Using a 250 mm 5 μm RP 18 column and acetonitrile–water (95:5, v/v) as mobile phase resulted in baseline separation of each of the investigated β-hydroxy acid methyl ester derivatives (Fig. 3).

The lowest detection limit (signal-to-noise ratio 3:1) was obtained for the anthroyl derivative (0.4 fmol). Amounts between 2 fmol [0.5 pg β-OH(14:0)] and 1 pmol [240 pg β-OH(14:0)] showed a linear correlation to the measured fluorescence signal. The comparison of the signal intensities was based on the detector response (area under the curve, unit: mVs).

Two fmol, 4 fmol, 8 fmol, 14 fmol, and 20 fmol of 3-(9-anthroyl)myristic acid ethyl ester, 3-(9-fluorenoyl)myristic acid ethyl ester, and 3-[4-(1-pyrenyl)butyroyl]myristic acid ethyl ester were separately dissolved in 150 μl acetonitrile. Signal intensities of this solutions were measured after HPLC separation at specific excitation and emission wavelengths for each fluorophore (Fig. 4). The fluorescence signal intensity of the anthroyl derivative was 7.4 times higher, the intensity of the 9-fluorenoyl derivative 2.5 times higher than that of the 4-(1-pyrenyl)butyroyl derivative. Therefore, all further studies were carried out using 9-anthroyl chloride as derivatizing agent.
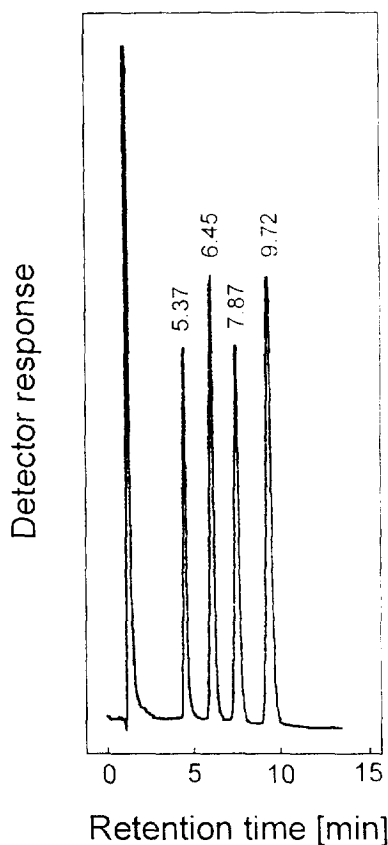
Fig. 3. Chromatogram showing peaks of 3-(9-anthroyl)lauric acid methyl ester (5.37 min), 3-(9-anthroyl)tridecanoic acid methyl ester (6.45 min), 3-(9-anthroyl)myristic acid methyl ester (7.87 min) and 3-(9-anthroyl)pentadecanoic acid methyl ester (9.72 min). Derivatization was carried out on a sample containing 10 ng each of β-hydroxy acid ethyl esters. HPLC: column, 5 μm RP 18, 250 × 3 mm I.D.; mobile phase, acetonitrile–water (95:5, v/v), 1 ml/min; sample loop, 10 μl; fluorescence detection at excitation (Ex) = 250 nm and emission (Em) = 462 nm.

3.2. Formation of β-hydroxy acid methyl esters from endotoxin

The literature-cited concentration of acid catalyst used for the liberation of β-hydroxymyristic acid from endotoxins for methanolysis prior to GC–MS analysis varies between 1 mol/l [21] and 4 mol/l [10], the duration of the heating between 1 h [19] and 18 h [10]. For optimization of both these reaction parameters, hydrochloric acid (1 mol/l and 4 mol/l) and sulfuric acid (0.5 mol/l
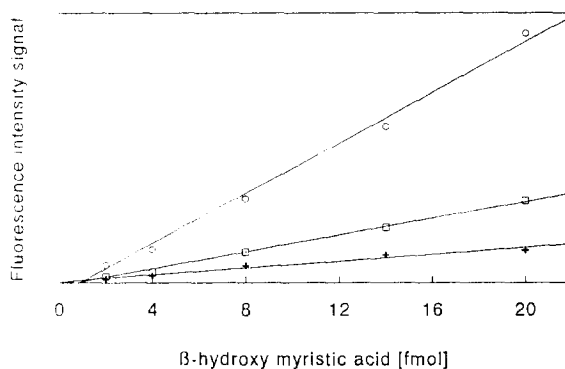

Fig. 4. Comparison of fluorescence signal intensities (average of three determinations) of 3-(9-anthroyl)myristic acid methyl ester (○: Ex = 250 nm, Em = 462 nm), 3-(9-fluorenoyl)myristic acid methyl ester (□: Ex = 262 nm, Em = 310 nm) and 3-(4-(1-pyrenyl)butyroyl)myristic acid methyl ester (+ : Ex = 238 nm, Em = 376 nm). HPLC conditions as in Fig. 3.

and 2 mol/l) were used. The samples with 100 ng endotoxin from E. coli 0111:B4 were heated for 0.5, 1, 2, 4 and 8 h. Fig. 5 shows the kinetics of the liberation of β-hydroxymyristic acid from endotoxin. β-Hydroxypentadecanoic acid was used as an internal standard. Recoveries were calculated from the fluorescence intensity/concentration ratio of the isolated derivatives.
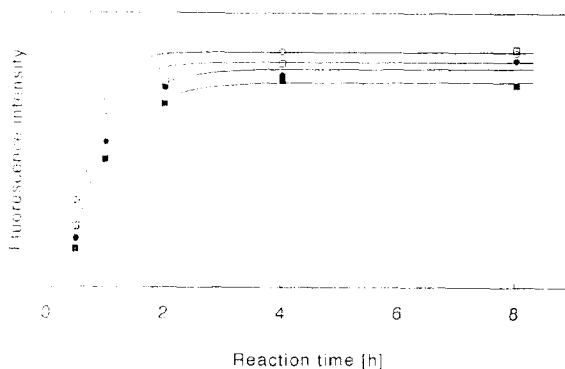
After 4 h, the reaction showed a steady state

Fig. 5. Kinetics of formation of free β-hydroxymyristic acid methyl ester from endotoxin of E. coli 0111:B4 with varying concentrations of hydrochloric acid (○: 4 mol/l; ●: 2 mol/l) and sulfuric acid (□: 2 mol/l; ■: 1 mol/l). β-Hydroxymyristic acid methyl ester was analyzed according to described procedure. HPLC conditions as in Fig. 3.

with all employed acid concentrations and the best results were obtained with sulfuric acid. Therefore, heating at 110°C for 4 h with 2 mol/l sulfuric acid was selected for methanolysis.

3.3. Separation of β-hydroxy acid methyl esters

After methanolysis, extraction with n-hexane, and solid-phase adsorption on Lichrolut Si cartridges, β-hydroxy (myristic and pentadecanoic) acid methyl esters were washed (4 ml), and eluted (2×700 μl) with increasing concentrations of tert.-butyl methyl ether in n-hexane. β-Hydroxy fatty acids were not eluted from the adsorbent at a tert.-butyl methyl ether concentration of 8% (v/v), but were completely eluted with 1 ml of a solvent containing 25% tert.-butyl methyl ether in hexane (Fig. 6a).

3.4. Removal of excess reagent

Because of the high ratio of excess reagent to product, the reaction mixture could not be employed directly for analysis in HPLC. For removal of excess reagent, the reaction mixture was allowed to adsorb on RP 18 cartridges. For optimization of cartridge washing, increasing acetonitrile concentrations were tested (Fig. 6b). Best results were obtained using 5 ml of a acetonitrile–water mixture (60:40, v/v) for washing. The product was eluted completely from the cartridge with 1 ml acetonitrile. The whole analysis method is summarized in Table 4.

3.5. Detection limits of endotoxins

Standard solutions with concentrations between 10 pg/ml and 10 ng/ml of each of the three investigated endotoxins were analyzed according to the optimized procedure. β-Hydroxypentadecanoic acid ethyl ester was used as an internal standard for quantitation of endotoxins with β-hydroxymyristic acid. Endotoxins may have a changing number of oligosaccharide units in their polysaccharide chain and do not posses a defined molecular mass [4]. The content of β-hydroxy acid varies, depending on the length of the polysaccharide chain. So, the different con-
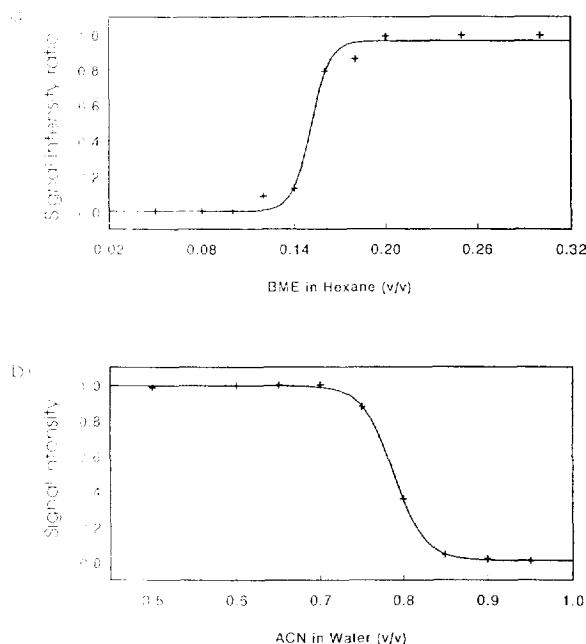

Fig. 6. (a) Elution profile of β-hydroxymyristic acid methyl ester from Lichrolut Si cartridges with increasing concentration of tert.-butyl methyl ether (BME) in hexane; elution volume: 2×700 μl. Signal intensity ratio was calculated from the signal intensities of eluted β-hydroxymyristic acid methyl ester before and after adsorption on cartridges. The ester concentration was 100 pg/ml, 387 fmol/ml) in hexane, the applied volume 1.5 ml. The ester was derivatized with 9-anthroylchloride (5 mg/ml in acetonitrile, 100 μl, 20 min at room temperature) after removal of hexane. HPLC conditions as in Fig. 3. (b) Elution of 3-(9-anthroyl)myristic acid methyl ester from RP 18 cartridges with increasing concentration of acetonitrile in water during the preelution procedure; 3-(9-anthroyl)myristic acid methyl ester (277 pg dissolved in 50 μl acetonitrile–water, 50:50) was applied to a cartridge and washed with 5 ml of varying acetonitrile concentrations in water. After preelution, remaining derivatives were eluted with 1 ml acetonitrile. HPLC conditions as in Fig. 3.

tents of β-hydroxymyristic acid were determined in the investigated lipopolysaccharides (LPS). β-Hydroxy acid content of the endotoxins from the smooth strain (*E. coli* 0111:B4) was only 32 μg β-OH(14:0)/mg LPS. Endotoxin from the rough strains (*Salmonella typhimurium* SL 1181 and *E. coli* Nissle 1917) contained more β-hydroxymyristic acid [154 μg β-OH(14:0)/mg LPS and 108 μg β-OH(14:0)/mg LPS], according to a reduced or missing polysaccharide chain. De-

Table 4
Summary of steps used in lipopolysaccharide determination

Step	Reagent/volume	Reaction time/reaction temperature
Methanolysis	4 M H₂SO₄ in methanol, 5 ml total volume	4 h/110°C
Extraction	with 1.5 ml hexane, after addition of 2 ml water	
Solid-phase extraction I	Si cartridges	
Washing I	tert.-butyl methyl ether-hexane 8:92, 4 ml	
Extraction I	tert.-butyl methyl ether-hexane 25:75, 2 × 700 μl	
Derivatization	9-anthroylchloride in acetonitrile (5 mg/ml), 100 μl	20 min, room temperature or 10 min 60°C
Hydrolysis of excess reagent	40 μl water	5 min, room temperature
Solid-phase extraction II	RP 18 cartridges, preconditioned with acetonitrile–water 60:40	adsorption time: 5 min
Washing II	acetonitrile–water 60:40, 5 ml	
Extraction II	acetonitrile, 1 ml	
HPLC analysis	(HPLC conditions see caption for Fig. 3)	

tection limits of endotoxins were 20 pg for smooth strain and 5 pg of rough strain endotoxin per sample (Fig. 7). These endotoxin amounts were detected successfully dissolved in 10 ml pyrogen-free water, if a vacuum centrifuge was used prior to analysis. No disturbing peaks at related retention times were found in pyrogen-free water and buffer solutions, investigated with the described method. Pyrogen-free bovine serum albumin (up to 1 mg/ml) did not affect the accuracy of the test.

3.6. Recovery

Endotoxin from *E. coli* 0111:B4 was dissolved in 0.05% aqueous solution of triethylamine and

diluted to give concentrations of 10 ng/ml and 1 ng/ml. Recoveries and reproducibility were determined from six-fold analysis of each solution (100 μl) with β-hydroxypentadecanoic acid ethyl ester as internal standard (100 pg/sample and 10 pg/sample). The measured recovery was 92.5 ± 6.4% (mean ± S.D.) for the 1 ng amount and 78.5 ± 8.8% for the 100 pg amount. The 100% value was determined from the purified 3-(9-anthroyl)pentadecanoic acid ethyl ester.

4. Discussion

The described results indicate a highly sensitive method for analysis of β-hydroxy acids used

Detector response

7.82
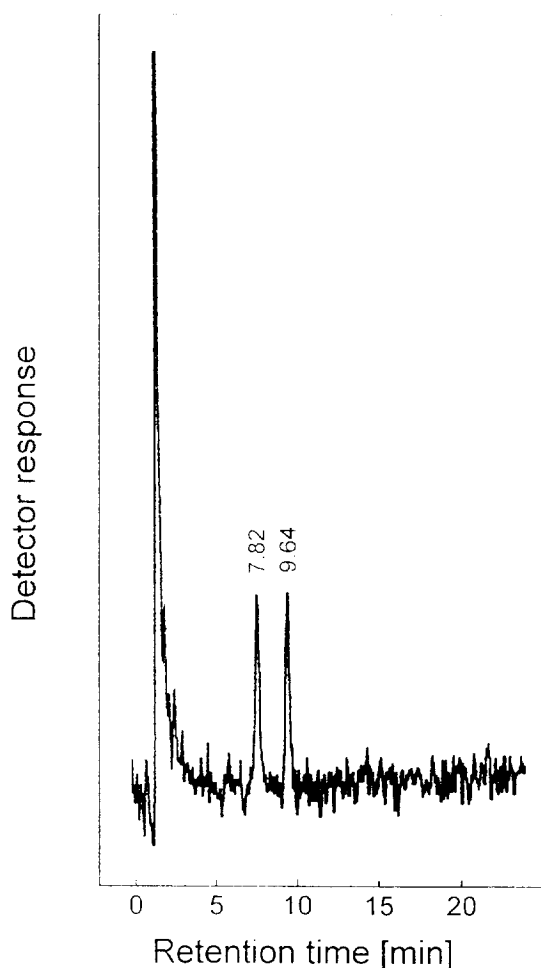9.64

0   5   10   15   20

Retention time [min]

Fig. 7. Chromatogram of 20 pg endotoxin from *E. coli* Nissle 1917 (7.82 min) and 2 pg β-hydroxypentadecanoic acid ethyl ester (9.64 min) after methanolysis and derivatization with 9-anthroyl chloride followed by twofold solid-phase extraction. HPLC conditions as in Fig. 3.

as markers for the determination of lipid A, the toxic component of lipopolysaccharides.

Analysis of specific microbial pyrogenic components is of special importance for investigation of compounds having contact with the systemic blood stream or tissue of mammals. The most common technique of measuring endotoxin content in aqueous solutions is the LAL test, an assay where lipid A activates the clotting cascade in a lysate from amebocytes from *Limulus poly-*

phemus [22]. Values for endotoxin contents in outer bacteria membranes determined by radioimmuno assays were about 10 times higher than the amounts measured with the LAL assay [23]. Further, false-negative results have been reported for the LAL assay as a result of inactivation of the enzymes of the clotting cascade [24]. Comparison of endotoxin contents in airborne dusts from poultry-processing industries by the LAL test and GC–MS analysis showed that the values determined by GC–MS were 10^2 to 10^5 above those obtained by LAL [9], calculated from the content of β-hydroxymyristic acid.

The discrepancy in these results is caused by the different approaches to the measurement of endotoxins. The LAL test is very sensitive (<5 pg/ml) [5], but rather measures a biological effect of endotoxins, which can be affected by physical, biological and chemical properties of the sample matrix [25,26]. Electrolytes, proteins and hormones for example may inactivate the clotting cascade of the LAL assay [24].

β-Hydroxy acids are essential for pyrogenity of endotoxins [22,27]. Hence, the absence of these acids is a sufficient evidence for the absence of pyrogens from Gram-negative bacteria. A further established technique for measuring the content of endotoxins is the determination via GC–MS [8–11,18], but the expensive equipment and the great demand on personnel qualification limits the application of this method.

The described method may be used to determine endotoxins in aqueous solutions, using β-hydroxy acids as markers, detecting lipopolysaccharides in both the free and the hidden state. Since proteins are destroyed by acid-catalyzed methanolysis, no "hiding" of endotoxins by protein adsorption can occur. Endotoxins can be quantitated by the described method even in the presence of proteins. In the presence of albumin (1 mg/ml), endotoxins could be still detected with the same precision. The applicability of this assay still has to be proved for other matrices.

With acetonitrile as a solvent there were no noticeable problems of adsorption on the used plastic- and glassware, even at sub-ng/ml con-

centrations. This fact can also be seen from the determined recovery at sub-ng/ml levels.

Contamination and memory effects were circumvented by using only single-use adsorption cartridges and heating glassware for 14 h at 300°C. After keeping glassware at this temperature, no contamination or memory effects occurred.

Although a normal-phase HPLC separation of β-hydroxy acids with adjacent aromatic polycyclic hydrocarbons has been described [15], we decided to develop a reversed-phase HPLC assay. During acylation with acyl chlorides, it is unavoidable that a part of the acyl chloride reacts with ubiquitous traces of water forming the free acid. This acid is adsorbed on the column during normal-phase HPLC, changing the surface properties of the stationary phase and making it difficult to obtain reproducible results. In reversed-phase chromatography, the polar carboxylic acid is eluted prior to the acylated products, so that no disturbance can occur during the following analysis.

Obtaining really dry acetonitrile and keeping it dry for and in routine applications is of essential interest carrying out derivatisations with carboxylic chlorides. Even commercially obtainable "ultra-dried" solvents contain about 50 ppm water. This amount, together with surface-adsorbed water on glass vials and air humidity reacts with the derivatizing agent. The reactivity of the hydrolyzed product, the free carboxylic acid, is too poor for formation of β-hydroxy acid methyl esters. Hence, acyl chloride concentrations of 1 μg/ml [15] proved to be too low for complete derivatization during routine analysis.

Apart from water, other reactive compounds such as alcohols and amines if present in the sample, can react with the acyl chloride making the derivatization incomplete. This makes it necessary to use excess derivatizing agent. In practice, a concentration of 5 mg/ml of anthroyl chloride showed the best results, making a visual control during analysis possible. The yellow color of the 9-anthroyl solution in acetonitrile faded to nearly colorless if the acid chloride was destroyed by the presence of reactive compounds. In this case, practically no acylation of

β-hydroxy acids could be observed. A further control of complete acylation is given by the use of an internal standard.

β-Hydroxylauric acid could also be detected by this assay (Fig. 2) with comparable detection limits, indicating the presence of *Neisseria meningitidis* [11]. Because of the high pathogenity of this microorganism and the lack of commercially available *Neisseria* endotoxin the applicability of this HPLC assay was not tested.

The catalysis of acylation with 9-anthroyl chloride by organoamine bases like pyridine or 4-dimethylaminopyridine has been reported to be unsuccessful [28], therefore no efforts were made to prove reaction enhancement by these compounds.

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References

[1] E. Rietschel, T. Kirikae, U. Schade, A. Ulmer, O. Holst, H. Brade, G. Schmidt, U. Mamat, H.-D. Grimmecke, S. Kusumoto and U. Zähringer, Immunobiol., 187 (1993) 169.

[2] C. Galanos, O. Lüderitz, E. Rietschel and O. Westphal, Int. Rev. Biochem. Biochemistry of Lipids II, (T. Goodwin, Editor), 14 (1977) 239.

[3] J. Hewett and R. Roth, Pharmacol. Rev., 45 (1993) 381.

[4] P. Friberger, in J. ten Cate, H. Büller, A. Sturk and J. Levin (Editors), Bacterial Endotoxins: Structure, Biomedical Significance and Detection with the Limulus Amebocyte Lysate Test, Alan R. Liss, New York, 1985, p. 139.

[5] H. Fukui, B. Brauner, J. Ch. Bode and Ch. Bode, J. Hepat., 12 (1991) 162.

[6] R. Elin and A. Utter, J. Clin. Microbiol., 12 (1980) 502.

[7] T. Mikami, T. Nagase, T. Matsumoto, S. Suzuki and M. Suzuki, Microbiol. Immunol., 26 (1982) 403.

[8] S.K. Maitra, R. Nachum and F.C. Pearson, Appl. Environ. Microbiol., 52 (1986) 510.

[9] A. Sonesson, L. Larsson, A. Schutz, L. Hagmar and T. Hallberg, Appl. Environ. Microbiol., 56 (1990) 1271.

[10] A. Sonesson, L. Larsson, R. Andersson, N. Adner and K.G. Tranberg, J. Clin. Microbiol., 28 (1990) 1163.

[11] P. Brandtzaeg, K. Bryn, P. Kierulf, R. Ovstebo, E. Namork, B. Aase and E. Jantzen, J. Clin. Invest., 89 (1992) 816.

[12] M. Bayliss and R. Homer, J. Chromatogr., 445 (1988) 393.

[13] H. Takamura and M. Kito, J. Biochem., 109 (1991) 436.

[14] J. Browning and D. Nelson, J. Membrane Biol., 49 (1979) 75.

[15] K. Tanamoto, Adv. Exp. Med. Biol., 256 (1990) 203.

[16] K. Nützel, Organo-Zink Verbindungen, Bd. XIII/2 Springer, Stuttgart, 1976, p. 809.

[17] O. Westphal and K. Jann, Meth. Carbohydr. Res., 63 (1965) 83.

[18] S.K. Maitra, M.C. Schotz, T.T. Yoshikawa and L.B. Guze, Proc. Natl. Acad. Sci. USA, 75 (1978) 3993.

[19] J. Parker, G. Smith, H. Fredrickson, J. Vestal and D. White, Appl. Environ. Microbiol., 44 (1982) 1170.

[20] A. Sonesson, L. Larsson and J. Jimenez, J. Chromatogr., 490 (1989) 71.

[21] A. Sonesson, L. Larsson, G. Westerdahl and G. Odham, J. Chromatogr., 417 (1987) 11.

[22] H. Takada, S. Kotani, S. Tanaka, T. Ogawa, I. Takahashi, M. Tsujimoto, T. Komuro, T. Shiba, S. Kusumoto, N. Kusunose, A. Hasegawa and M. Kiso, Eur. J. Biochem., 175 (1988) 573.

[23] R. Munford and C. Hall, in S. Watson, J. Levin and T. Novitsky (Editors), Detection of Bacterial Endotoxins with the Limulus Amebocyte Lysate Test, Alan R. Riss, New York, 1987, p. 93.

[24] A. Weiss, in S. Watson, J. Levin and T. Novitsky (Editors), Detection of Bacterial Endotoxins with the Limulus Amebocyte Lysate Test, Alan R. Riss, New York, 1987, p. 235.

[25] D. Morrison, S. Vukajlovich, J. Ryan and J. Levin, in S. Watson, J. Levin and T. Novitsky (Editros), Detection of Bacterial Endotoxins with the Limulus Amebocyte Lysate Test, Alan R. Riss, New York, 1987, p. 55.

[26] F. Pearson, R. Caruana, J. Burkart, D. Katz, D. Chenoweth, J. Dubczak, J. Bohon and M. Weary, in S. Watson, J. Levin and T. Novitsky (Editors), Detection of Bacterial Endotoxins with the Limulus Amebocyte Lysate Test, Alan R. Riss, New York, 1987, p. 211.

[27] J. Homma, M. Matasura, S. Kanegasaki, Y. Kawakubo, Y. Kojima, N. Shibukawa, Y. Kumazawa, A. Yamamoto, K. Tanamoto, T. Yasuda, M. Imoto, H. Yoshimura, S. Kusumoto and T. Shiba, J. Biochem., 98 (1985) 395.

[28] M. Bayliss, R. Homer and M. Shepherd, J. Chem. Soc., Chem. Commun., (1990) 305.