Quantification of bacterial lipopolysaccharides (endotoxin) by GC–MS determination of 3-hydroxy fatty acids

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A GC–MS method for the quantification of bacterial lipopolysaccharides (LPS, endotoxin) is presented. After hydrolytic cleavage of 3-hydroxy fatty acids (3-OH FAs) from the lipid A region of LPS, derivatisation of both the hydroxyl and the carboxyl group was performed in one step with a mixture of methyl-bis(trifluoracetamide) (MBTFA) and N-methyl-N-(tert-butylidimethylsilyl)trifluoroacetamide (MTBSTFA). Using GC–MS in the EI mode with selected ion monitoring (SIM) for analysis, baseline separation of 3-OH FAs (and of possibly interfering 2-OH FAs) was achieved. The sensitivity of the method (LOD 7–50 pg/injection for the different 3-OH FAs investigated) allows for the efficient quantification of LPS in occupational and environmental samples. Degradation of 3-OH FAs as well as of their derivatives during sample preparation and GC–MS separation as a possible source of errors in analytical methods based on 3-OH FA determination is reported for the first time. Thermal elimination of water from the underivatised 3-OH FAs and of trifluoroacetic acid from the derivatives was identified as the cause of degradation. The resulting α,β-unsaturated compounds showing the same mass spectra as the 3-OH FA derivatives were detected as more or less prominent satellite peaks. By using alkaline instead of acidic hydrolysis and cool on-column instead of split/splitless injection, elimination was reduced to an acceptable level.

Introduction

Endotoxins represent a widespread occupational and environmental hazard, since their biological activity is independent of the living bacteria, it persists even after cell death. Thus they are found in dusts of any origin, from generally only moderately contaminated dust in private homes, schools, pig farms, poultry farms, and aircrafts to dusts from areas with a high microbial and/or fungal load such as hospitals, animal feed, and industrial plants. Endotoxins are ubiquitous molecules which–together with the corresponding polysaccharides–are the essential surface components of gram-negative bacteria. In chemical terms, endotoxins are lipopolysaccharides (LPS) with a terminal lipid region (lipid A) and a polysaccharide chain consisting of the O-specific chain (O-antigen) and the core oligosaccharide. While the structure of the core region is fundamentally identical for different bacteria, the O-specific chain, characterized by repeating oligosaccharide units of up to five saccharides, differs considerably between species. The O-antigen is the essential structure for the immune response and the formation of antibodies after infection. On the other hand, LPS remains biologically active after bacteriolysis and is responsible for the severe symptoms occurring in bacteraemia or sepsis, for example. The component responsible for the toxic effect of LPS is lipid A: polysaccharide-free lipid A obtained by hydrolysis of LPS shows the same activity in test systems as the complete LPS. A characteristic feature of lipid A is that it contains ester- or amide-bonded 3-hydroxy fatty acids (3-OH FAs). Depending on the bacterial species, lipid A is characterized by different 3-OH FAs with chain lengths of 10, 12, 14, 16, 18, or more carbon atoms. As an example, 1 mol of lipid A from E. coli contains 4 mol of 3-hydroxytetradecanoic acid (14:0 3-OH FA).

Endotoxins are proinflammatory substances leading to a release of mediators of inflammation such as TNF-α and interleukins (IL-1β, IL-6, IL-8). Inhaled endotoxin may lead to bronchial hyperresponsiveness, fever or nausea. Deleterious effects on the clinical severity of chronic asthmatic disease have been found after domestic exposure to endotoxin-contaminated house dust. With respect to occupational exposure, significant associations between endotoxin concentrations and restrictions of lung function have been found in various cross-sectional studies, while other studies failed to confirm these findings. These contradictory results might be due to the heterogeneity of inhaled dusts, since a variety of components (e.g. fungi) might contribute to the observed health effects. On the other hand, endotoxin quantification in these studies might be erroneous. The Limulus amebocyte lysate assay (LAL) currently used for endotoxin determination was first described by Levin and Bang. The LAL test monitors the biological activity of endotoxins and is based on a gelation reaction of the lysate in the presence of endotoxin. But, though it is very sensitive, the assay is subject to various interferences. Hollander and coworkers attributed the inhibition of the LAL assay in samples from the animal-feed industry to vitamins, antibiotics, and minerals which are added to raw materials in the pre-mix department. An enhancement of the assay was found in samples from the unloading department of the same plant, which was explained by specific activation of the LAL proclotting enzyme, e.g. by β-glucans. β-Glucans originating from plants or mould are known to enhance LAL activation. Furthermore, the LAL test exhibits...
differing sensitivity to LPS from different bacteria and seems to underestimate the endotoxin content of a sample compared to the results of endotoxin determination by radioimmunoassay.

Because of these drawbacks of the LAL test, various attempts have been made to establish alternative methods for endotoxin determination based on chemical analysis. In the present paper, a method suited for the determination of endotoxins in aqueous samples as well as in samples of settled or airborne dust is presented. It is based on the derivatisation technique used by Costa et al. for the analysis of fatty acids in plasma. After hydrolytic cleavage of the LPS 3-OH FAs they are derivatised in a one-step reaction using a mixture of N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide (MTBSTFA) and methyl-bis(trifluoroacetamide) (MBTFA). MBTFA selectively reacts with the hydroxyl functionality, and MTBSTFA with the carboxyl group. GC–MS is used for separation and quantification.

Experimental

Apparatus
Gas chromatograph 5890 Series II with mass selective detector MSD 5971 and injector 6890 (Agilent Technologies); capillary column Ultra-2 (Agilent Technologies, 50 m × 0.2 mm, film thickness 0.33 µm) for split/splitless injection; capillary column HP-1701 (Agilent Technologies, 30 m × 0.25 mm, film thickness 0.25 µm) for cool on-column injection with an additional deactivated capillary (2 m × 0.53 mm) coupled to the inlet side of the column as retention gap; carrier gas: helium; rotating mixer for derivatisation (own construction) to guarantee close contact of derivatising agents with 3-hydroxy fatty acids precipitated at the vial walls during sample preparation (see below); pyrogen-free Multi fit Guard Filter Tips (IVA Analysentechnik); glass vials 1.5 ml with aluminium caps (IVA Analysentechnik); glass test tubes with screw caps; vials and test tubes were made pyrogen-free by heating them to 300 °C for 4 h before use.

Chemicals
2-Hydroxyoctanoic acid (8:0 2-OH), 2-hydroxydecanoic acid (10:0 2-OH), 2,3-dihydroxydecanoic acid (12:0 2-OH), 2-hydroxytetradecanoic acid (14:0 2-OH), 3-hydroxydecanoic acid (10:0 3-OH), 3-hydroxydecanoic acid (12:0 3-OH), 3-hydroxytetradecanoic acid (14:0 3-OH), 3-hydroxyhexadecanoic acid (16:0 3-OH) (all from Sigma), trans-2-decanoic acid (10:1) (Acros), lipopolysaccharide E. Coli (Sigma, lot 069H4046, activity: 3000 000 endotoxin units mg⁻¹), methyl-bis(trifluoroacetamide) (MBTFA) (Sigma), N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide (MTBSTFA), pyridine, ethyl acetate, chloroform, hydrochloric acid, sodium hydroxide, sodium sulfate (all from Merck), pyrogen-free water (Delta Pharma).

Solutions
Stock solutions of 2- and 3-hydroxy fatty acids in chloroform (250 µmol ml⁻¹), LPS stock solution in pyrogen-free water (1 mg ml⁻¹), 4 M hydrochloric acid, 8 M hydrochloric acid, 8 M sodium hydroxide, 16 M sodium hydroxide, saturated sodium chloride solution

Sample preparation and derivatisation
Derivatisation of free 2- and 3-hydroxy fatty acids: 20–100 µl from the stock solution of each fatty acid to be derivatised were pipetted into a glass vial. The solvent was removed with a gentle stream of nitrogen at room temperature. 50 µl MBTFA, 40 µl MTBSTFA, and 10 µl pyridine were added, resulting in a reaction solution containing 50–250 pmol µl⁻¹ of each fatty acid. The vials were capped and placed in the rotating mixer. The walls and base of the vial were continuously moistened with the agents by slow rotation of the mixer. Derivatisation was complete after 1 h at 60 °C. 1 µl of the solution was used for GC–MS analysis.

Hydrolysis of LPS
Acidic hydrolysis: In a test tube, 500 µl of 8 M HCl were added to 500 µl of LPS stock solution, thus resulting in a reaction solution of 4 M HCl. The tube was capped and then heated to 90 °C for 2, 4, 6, 8, or 24 h, respectively. After cooling down to room temperature, 1 ml saturated NaCl solution was added. The hydrolysate was extracted twice with 3 ml ethyl acetate. The combined organic phases were dried with 500 mg Na2SO4, transferred to a vial in portions from which the solvent was continuously removed with a gentle nitrogen stream at room temperature. Derivatisation of the dried extract was performed as described above.

Alkaline hydrolysis
Alkaline hydrolysis was tested for 4 M and for 8 M NaOH. In a test tube, 500 µl of 8 M NaOH (or 16 M NaOH) were added to 500 µl of LPS stock solution (reaction solutions of 4 M or 8 M NaOH, respectively). The tube was capped and then heated to 90 °C for 2, 4, 6, 8 or 24 h, respectively. After cooling down to room temperature, the solution was acidified with 1.1 ml of 4 M (or 8 M) HCl. 1 ml saturated NaCl solution was added, the hydrolysate then was extracted twice with 3 ml ethyl acetate. The combined organic phases were dried with 500 mg Na2SO4, transferred to a vial in portions from which the solvent was continuously removed with a gentle nitrogen stream at room temperature. Derivatisation of the dried extract was performed as described above.

GC–MS separation of derivatives with split/splitless injection: injector temperature: 280 °C; oven: initial temperature 90 °C, initial time 2 min, temperature gradient 5 °C min⁻¹; final temperature 280 °C, final time 15 min; column head pressure: 100 kPa; MSD detector: transfer line temperature: 180 °C, mass range mz 50–400.

Total ion chromatograms (TIC) were recorded to obtain mass spectra of the derivatives.

For quantitative analysis, detection was performed in the selected ion monitoring mode with the following parameters: mz 199: 18–22 min, mz 227: 22–30 min, mz 255: 30–35 min, mz 283: 35–39 min, mz 311: 39–43 min

GC–MS separation of derivatives with cool on-column injection: injector temperature: always 3 °C ahead the oven temperature (oven track mode), initial temperature: 93 °C; oven: initial temperature 90 °C, initial time 2 min, temperature gradient 5 °C min⁻¹ to 230 °C, hold time 2 min, temperature gradient 5 °C min⁻¹, final temperature 280 °C, final time 15 min; column head pressure: 100 kPa; MSD detector: transfer line temperature: 180 °C, mass range mz 50–400

Total ion chromatograms (TIC) were recorded to obtain mass spectra of the derivatives.

For quantitative analysis, detection was performed in the single ion monitoring mode with the following parameters: mz 199: 12–16 min, mz 227: 16–21 min, mz 255: 21–25 min, mz 283: 25–28 min, mz 311: 28–31 min

Results
Hydroxy fatty acids are readily derivatised using the procedure developed by Costa et al. for the analysis of fatty acids in plasma. In this procedure derivatisation is performed in a single step with a reagent mixture of MBTFA selectively reacting with the hydroxyl functionality and MTBSTFA reacting with the carboxyl group (Fig. 1). Mass spectra of the derivatives of 3-OH FAs (and of 2-OH-FAs) exhibit a common characteristic: Loss of the tert-butyl group after elimination of trifluoro acetic acid.
results in a fragment of M − 171 forming the base peak. Molecule peaks are not detected. This is exemplified for 14:0 3-OH FA in Fig. 2.

GC separation of derivatives with split/splitless injection was performed using the M − 171 fragment for mass selective detection with selected ion monitoring. Fig. 3 shows a chromatogram of the four 3-OH FAs investigated. This chromatogram shows that, under the selected GC conditions, all four derivatives showed satellite peaks detected with the same m/z for SIM as the main peaks. Furthermore, mass spectra of main and satellite peaks were identical. These satellites may have resulted either from side products or from degradation reactions. The hypothesis of degradation is supported by peak tailing or an elevated baseline between the main peak and its satellite, thus pointing to an on-column conversion of products. Further proof for this is provided by investigations into the dependence of peak ratios on injector temperature. The main peak continuously decreases with increasing injector temperature, while the satellite peak grows simultaneously.

Thermal decomposition of the derivatives is thus the cause of degradation occurring at high injector temperatures, leading to the sharp satellite peak. Additional on-column conversion then results in molecules with intermediate retention times leading to peak tailing. It was assumed that trifluoroacetic acid might be eliminated in this case as observed for 18:0 3-OH.23 The resulting MTBSTFA derivatives of the corresponding α,β-unsaturated fatty acids should show at least similar mass spectra, since elimination of trifluoroacetic acid is part of the fragmentation reaction of the 3-OH-FA derivatives in the ion source. As a final proof for these assumptions, trans-decenoic acid (10:1 FA) was derivatised, leading to a peak with an identical retention time and mass spectrum to the 10:0 3-OH FA satellite. These results suggest a reduction of the thermal load to avoid degradation of derivatives. Therefore cool on-column injection at comparably low temperatures was a promising alteration of the separation procedure. Fig. 4 shows a chromatogram of 3-OH FA derivatives after changing to cool on-column injection at 93 °C. Obviously, thermal degradation is reduced by magnitudes (note: retention times of main and satellite peak are reversed compared to split/splitless injection because of the different capillary column used).

With this improved separation, calibration of 10:0, 12:0, 14:0 and 16:0 3-OH FA was performed using 8:0 2-OH FA as internal standard. All calibration graphs were non-linear. This non-linearity could not be overcome by varying the method and may be due to the persisting thermal on-column degradation of derivatives. This minor disadvantage was judged to be acceptable, since the graphs provide a perfect fit with second-order equations which may be used for quantification:

$$y = 0.160 + 0.00397x + 4.06E^{-5}x^2, \quad R^2 = 0.9989;$$
Acidic hydrolysis of LPS of \( \text{E. coli} \), was detected in GC–MS analysis. Unfortunately the method is the method of choice for the liberation of 3-OH FAs from lipid A.

### Discussion

For the determination of bacterial LPS in occupational and environmental samples, the LAL assay still is the gold standard. But, though it is very sensitive, the assay is subject to various interferences,\(^\text{27}\) which is why various attempts have been made to develop alternative methods based on chemical analysis. All of these methods focus on the quantification of 3-hydroxy fatty acids from the lipid A of LPS. The basic steps are the same for all methods: after hydrolytic cleavage of the 3-OH FAs and derivatisation of the hydroxy and the carboxyl functionality, the derivatives are separated chromatographically.

The derivatising agents are chosen with respect to the intended separation and quantification techniques. HPLC with fluorescence detection was used after coupling a fluorophore (anthracene-9-carboxyl chloride, 9-fluorene carboxyl chloride or 4-(1-pyrenyl)butyric acid chloride) to the hydroxy group of the 3-OH FA methyl esters.\(^\text{28,29}\) Separation and quantification of halogen-containing 3-OH FA derivatives was performed using gas chromatography with electron capture detection. 3-OH FA pentafluorobenzyl esters, 3-O-heptafluorobutyryl-, 3-O-pentafluorobenzoyl- and 3-O-trichloroacetyl derivatives of 3-OH FA methyl esters as well as 3-O-heptafluorobutyryl derivatives of 3-OH FA pentafluorobenzyl or trichloroethyl esters have been investigated.\(^\text{30,31}\) Gas chromatography–mass spectrometry has been used by various groups for different derivatives: 3-O-trimethylsilyl-derivatives of 3-OH FA pentafluorobenzyl\(^\text{32}\) or methyl esters,\(^\text{3,33–37}\) 3-O-tert-butyldimethylsilyl derivatives of 3-OH-FA methyl esters\(^\text{38}\) and the above-mentioned halogenated derivatives used for electron capture detection.\(^\text{30,34,35,39}\)

One main advantage of the method presented in this paper is that derivatisation of both functionalities of the 3-OH FAs is conducted in a single step with no further clean-up. In most of the methods mentioned above, a two-step derivatisation reaction with more or less elaborate clean-up procedures is needed. Thus, the simplicity of the MBTFA/MTBSTFA method leads to a reduction in potential analytical errors during sample preparation. Furthermore, since 2-OH FAs might be present in occupational or environmental dust samples, their separation from 3-OH FAs is a basic prerequisite for quantification. While baseline separation of 2- and 3-OH FA derivatives is assured with MBTFA/MTBSTFA derivatisation, it could not be achieved\(^\text{36}\) or was not investigated in some of the other methods.

One problem which was addressed neither for any of the above mentioned methods nor in the investigations of Costa et al.\(^\text{28}\) is the tendency of 3-OH FAs to eliminate water during hydrolysis to result in \( \alpha,\beta \)-unsaturated fatty acids which then are derivatised to the corresponding MBTFB derivatives. Additional \( \alpha,\beta \)-unsaturated derivatives are formed during injection and separation of the derivatives by thermal
elimination of trifluoroacetic acid. This phenomenon has been observed by Costa et al.\(^\text{26}\) for the MBTFA/MTBSTFA derivative of 18:0 3-OH FA, but not for the short-chained 3-OH FA. These elimination reactions may have considerable influence on the analytical results. Depending on the split/ splitless injector temperature, substantial amounts of the derivatives are converted to the respective \(\alpha,\beta\)-unsaturated compounds. Additional on-column conversion, though less pronounced, increases the amount of elimination products. By changing to cool on-column injection, thermal degradation can be reduced effectively.

The formation of \(\alpha,\beta\)-unsaturated fatty acids from 3-OH FAs already during acidic hydrolysis of LPS represents a major problem. Changing to cold on-column injection, thermal degradation can be reduced effectively. However, with LPS of different species and thus with different 3-OH FAs, the absolute amounts of LPS cannot be considered as a possible side reaction when acidic hydrolysis is used or when methanolysis was applied for the liberation of 3-OH FAs as their methyl esters; elimination products might have not been separated from the derivatives under the selected chromatographic conditions; elimination products might have been overlooked when mass spectrometric detection with selected ion monitoring was used. But, whatever the reason, the results presented here indicate that elimination should always be considered as a possible side reaction when acidic hydrolysis of LPS is applied.

Though elimination could not be suppressed completely with alkaline hydrolysis, it is nevertheless the method of choice for the liberation of 3-OH FAs from LPS, since the yields of elimination products are low and independent of hydrolysis time.

Calibration using 8:0 2-OH FA as internal standard resulted in non-linear calibration graphs for all four 3-OH FAs investigated. Nevertheless, since a perfect fit to second-order equations is given, results may be calculated using these functions. Relative standard deviations at a concentration of 10 pmol \(\mu\)l\(^{-1}\) were from 6.81 to 14.46\(\%\), increasing with chain length. This increase may be due to a different tendency of 3-OH FAs to eliminate water (or trifluoroacetic acid): trifluoroacetic acid elimination was observed only for the long chain 18:0 3-OH FA.\(^\text{26}\) With limits of detection from 50–7 pg/injection or 5–0.7 ng/sample, the MBTFA/MTBSTFA method matches the sensitivity of the other methods based on 3-OH FA derivatisation. For the LPS of \(E.\) coli with a molecular mass of about 4000\(^\text{46}\) and four moles of 14:0 3-OH FA per mole, the LOD of 3-OH FA corresponds to 4.9 ng LPS/sample or about 15 endotoxin units (EU) for the LPS lot (3000000 EU \(\mu\)g\(^{-1}\)) used in these investigations. If necessary, the sensitivity may be improved tenfold by reducing the injection volume to a maximum of 5 \(\mu\)l and by increasing the injection volume to a maximum of 5 \(\mu\)l.

The reliability and sensitivity of the MBTFA/MTBSTFA method thus meet all requirements for the quantification of endotoxin concentrations usually found in occupational\(^\text{61}\) or environmental\(^\text{62}\) dust samples.

Conclusions

GC–MS determination of 3-hydroxy fatty acids from lipid A of bacterial lipopolysaccharides provides quantitative information about the endotoxin content in aqueous or dust samples. However, it must be borne in mind that the results obtained are completely different from those of the LAL assay. Endotoxin measurement with the LAL assay reflects the overall biological activity of a sample in this specific test system, which must not necessarily be correlated with the activity or toxicity in vivo. On the other hand, with LPS of different species and thus with different 3-OH FAs, the absolute amounts of LPS cannot be deduced from the results of the MBTFA/MTBSTFA method (or of any other method based on 3-OH FA quantification). With these differences it is hardly surprising that correlations between LAL and 3-OH FA based results are usually poor. The best correlation coefficients (0.57–0.61) are obtained when only 10:0, 12:0 and 14:0 3-OH FAs are considered individually or in combination.\(^\text{3}\)

The quantitative information about the endotoxin content delivered from 3-OH FA based methods thus represents—compared to the LAL results—an analytical result sui generis. Further investigations on the association of health effects with endotoxin exposure assessed either by the LAL or by the MBTFA/MTBSTFA method are needed to decide which is the method of choice for the comprehensive characterization of endotoxin.

References
