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Profiling of 3-hydroxy fatty acids as environmental markers of endotoxin using liquid chromatography coupled to tandem mass spectrometry

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ABSTRACT

3-Hydroxy acids are constituents of the lipid A part of lipopolysaccharides and may potentially be used as chemical markers of endotoxin. While commercial enzymatic assays, such as the widely used *Limulus* amebocyte lysate (LAL) assay, commonly detect merely the water-soluble fraction of the bioactive endotoxin, the chemical approach aims to estimate the total amount of endotoxin present in a sample. Our objective was to develop a simple method for quantitative profiling of 3-hydroxy fatty acids in occupational and environmental samples based on detection with HPLC–MS/MS. We included eleven 3-hydroxy fatty acids (3-hydroxyoctanoic acid to 3-hydroxyoctadecanoic acid) in the HPLC–MS/MS based method, which involved base hydrolysis of filter samples using 1 M sodium hydroxide and removal of the base as well as concentration of the fatty acids using solid-phase extraction on a functionalized polystyrene-divinylbenzene polymer. Recovery trials from spiked glass fiber filters, using *threo*-9,10-dihydroxyhexadecanoic acid as internal standard, gave an overall recovery of 54–86% for 3-hydroxy fatty acids of medium chain length (3-hydroxynonanoic to 3-hydroxypentadecanoic acid). 3-Hydroxyoctanoic acid and the longer chain fatty acids were more problematic yielding overall spike recoveries of 11–39%. While the 3-hydroxy fatty acid profile of pure lipopolysaccharides was dominated by 3-hydroxydecanoic, 3-hydroxydodecanoic and 3-hydroxytetradecanoic acid the aqueous phase from drilling mud contained in addition relatively high amounts of 3-hydroxyoctanoic and 3-hydroxynonanoic acid. Endotoxin activity as measured by the LAL assay was reasonably correlated ($R^2 = 0.54$) to the sum of 3-hydroxydecanoic acid, 3-hydroxydodecanoic acid and 3-hydroxytetradecanoic acid in these samples.

1. Introduction

The discovery of endotoxin dates back to the end of the 19th century when a cell-associated material from *Vibrio cholerae* was found to induce toxic reactions in guinea pigs [1]. The pathophysiological symptoms associated with endotoxin include fever, diarrhoea, septic shock and death [2]. However, symptoms following the inhalational exposure to endotoxin include a local and a systemic inflammatory response at lower doses of lipopolysaccharide, while higher inhaled doses are required to elicit significant clinical and lung function responses [3]. Endotoxins are commonly found in different workplaces such as in animal farming, agricultural work and the waste industry [4]. Endotoxin is part of the cell-membrane of Gram-negative bacteria and consists of lipopolysaccharide, which is composed of three structurally different parts: the glycolipidic part, named lipid A, a polysaccharide core, and an O-specific chain [5]. Lipid A has been recognized as the 'endotoxic principle', i.e., it is the part of lipopolysaccharide that is linked to its toxic properties [6]. Lipid A is the most conserved part of the molecule and consists of a diglucosamine backbone substituted with four to seven ester- or amide-linked fatty acids [5,7]. There are certain structural requirements for lipid A being biologically active such as two glycosidically linked hexosamine residues, two phosphoryl groups and six fatty acids [6]. Another important aspect for the biological activity of lipopolysaccharides is the molecular conformation of single molecules and their type of aggregation [8,9].

The exposure to airborne endotoxin is presently most often assessed using the *Limulus* amebocyte lysate (LAL) test in its kinetic

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chromogenic version [10]. Although the assay is highly sensitive, several critical aspects have been reported in the literature. These are (1) reduced bioactivity of lipopolysaccharide in the assay because of the possible formation of superstructures or micelles due to its amphiphilic character, (2) the cross-reactivity of the LAL assay with several other types of biomolecules such as β-D-glucans and (3) the possible underestimation of endotoxin because the assay merely detects the water soluble fraction of endotoxin [10,11].

A measure of the total endotoxin in a sample can be obtained from chemical analysis of free 3-hydroxy fatty acids after hydrolysis of lipopolysaccharide [12–15]. The determination of 3-hydroxy fatty acids can thus also be useful for comparing the water soluble versus the water insoluble fraction of endotoxin as well as for optimising sample treatment and extraction [13]. It is, however, difficult to interpret the results from 3-hydroxy fatty acid analysis, as their concentrations cannot directly be related to biological effects. As a result, the measured 3-hydroxy fatty acid concentrations correlate commonly rather poorly to the endotoxin activities measured in the LAL assay [16].

According to the literature, the by far most often used chemical method for the analysis of lipopolysaccharide-related 3-hydroxy fatty acids consists of methanolysis of samples followed by acylation of the hydroxyl-group and instrumental analysis using gas chromatography coupled to mass spectrometry (GC–MS) [12–16]. Recently, the use of liquid chromatography coupled to triple quadrupole mass spectrometry (HPLC–MS/MS) for the determination of two 3-hydroxy fatty acids has been reported in this journal [17]. The protocol was straightforward, including base hydrolysis of lipopolysaccharide using 2 M sodium hydroxide, liquid/liquid extraction after neutralisation and subsequent instrumental analysis. However, the recoveries of the two 3-hydroxy fatty acids in the liquid/liquid extraction step were rather low, and the method was not designed for analysis of environmental samples. Our intention was thus to develop a simple and fast method for the quantitative profiling of 3-hydroxy fatty acids in environmental samples based on HPLC–MS/MS for (1) optimisation of sample treatment and extraction of endotoxin and (2) alternative and complementary analysis of endotoxin exposure at workplaces and other environments.

2. Material and methods

2.1. Chemicals and reagents

HPLC-grade acetonitrile and methanol was from Rathburn Chemicals Ltd., (Walkerburn, Scotland). Water for LC–MS was purified and deionised using a Millipore Elix 5/Milli-Q Academic water purification system (Merck Millipore, Merck KGaA, Darmstadt, Germany), while LAL reagent water was from Lonza (Basel, Switzerland). Ammonium formate (97%), formic acid (98%, pro analysis grade), sodium hydroxide (2 M) and α-cyclodextrin (purum, >98%) as well as lipopolysaccharides from *Escherichia coli* 0111:B4, *Klebsiella pneumonia*, *Pseudomonas aeruginosa* 10 and *Serratia marcescens* were from Sigma–Aldrich (St. Louis, MO, USA). Sodium carbonate and sodium bicarbonate (both p.a. quality) were from Merck. Tween-20 was obtained as a 10% solution in water in 10-mL-ampoules (Life Technologies, Carlsbad, CA, USA). 3-Hydroxy fatty acids (>99%, chain length C8–C18) as well as a racemic mixture of *threo*-9,10-dihydroxyhexadecanoic acid (>99%) were obtained from Larodan (Solna, Sweden).

2.2. Preparation of calibration solutions

All standard stock and calibration solutions were prepared in methanol/water (4:1, v/v) and stored in a freezer (−20 °C). Stock solutions of individual 3-hydroxy fatty acids were prepared at a concentration of 100 μg/mL and aliquots combined to a working stock solution containing 1 μg/mL of each 3-hydroxy fatty acid. Calibration solutions for six-point calibration plots were prepared in the range 2.5–1000 ng/mL, each containing 100 ng/mL of *threo*-9,10-dihydroxyhexadecanoic acid as internal standard.

2.3. Sampling at drilling mud recycling plants and preparation of filter samples

Mud from oil drilling was manually collected from storage basins at specialised recycling plants in the Kristiansund area of Norway and transported in 100-mL polyethylene bottles. Aliquots of approximately 5 mL were centrifuged at 2000 × *g* for 15 min and 100 μL of the water phase transferred to glass fiber filters (25-mm i.d.; Whatman GF/A, GE Healthcare, Buckinghamshire, UK). The filters were dried under a stream of nitrogen before being either extracted for LAL assaying or base hydrolysis.

At the same plants, air samples were collected with a flow rate of 2 L/min through Personal Air Samplers with a 6 mm wide orifice (PAS-6, University of Wageningen, The Netherlands) containing 25 mm Whatman GF/A filters. The flow was maintained for 2–6 h depending on the time that was available during the visits, and sampling sites selected such that important workspaces were covered. All GF/A filters were heated to 400 °C for 6 h before sampling.

2.4. Hydrolysis and solid-phase extraction

Round-bottom glass tubes (100 × 16 mm, VWR International, Oslo, Norway) were heated to 400 °C for 6 h. The filters were transferred to the tubes and 20 μL of a 1000 ng/mL solution of the internal standard added. The filters were dried under a stream of nitrogen and subsequently, 1 mL of pyrogen free water, containing 0.1% Tween-20, and 1 mL of 2 M NaOH were added. The tubes were heated to 90 °C for 30 min. They were then allowed to cool to room temperature, and the entire liquid phase applied to Strata-X columns (60 mg, Phenomenex, Torrance, CA, USA), which had previously been conditioned with 1.2 mL of methanol followed by 1.2 mL of water. After application of the hydrolysate, the columns were washed with 2 × 1.2 mL of water followed by 1.2 mL of water containing 1% of methanol and then dried under vacuum for 1 min. The columns were eluted with 1.2 mL of methanol. The methanol was evaporated at 60 °C, using a gentle stream of nitrogen. Residues were dissolved in 0.2 mL of methanol/water (4:1, v/v) by vortexing followed by sonication for 10 min. The solutions were transferred to screw-cap chromatography vials and stored in the refrigerator at 4 °C until instrumental analysis.

2.5. HPLC–MS/MS

HPLC was performed on a 75 × 2.1 mm Kinetex C18 column (2.6 μm particles; Phenomenex) fitted to an UltiMate 3000 RS UHPLC system equipped with autosampler (Thermo Fisher Scientific, Waltham, MA). The column was maintained at 30 °C, and elution was at 0.4 mL/min. Mobile phase A was water containing 5 mM ammonium formate and 0.1% of formic acid. Mobile phase B was prepared by dissolving 5 mM ammonium formate and 0.1% of formic acid in 50 mL of water and adding acetonitrile to a final volume of 1 L. Separation was performed by eluting the column isocratically with 10% B for 0.5 min and then changing the mobile phase composition linearly to 100% B over 14.5 min. The column was then flushed with 100% B for 2 min, returned to the starting conditions and equilibrated for 3 min. Under these conditions, the 3-hydroxy fatty acids eluted between 4 and 12.8 min (Fig. 1).

The UHPLC was coupled to a TSQ Vantage EMR triple quadrupole mass spectrometer (Thermo Fisher Scientific) equipped with a