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β-Hydroxymyristic acid as a chemical marker to detect endotoxins in dialysis water

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ABSTRACT

An analytical chemical method has been developed for determination of β-hydroxymyristic acid (β-HMA), a component of lipopolysaccharides (LPSs/endotoxins) in dialysis water. In our investigation, the β-HMA component was used as a chemical marker for endotoxin presence in dialysis water because it is available in the molecular subunit (lipid A) and responsible for toxicity. It is the most abundant saturated fatty acid in that subunit. The developed method is based on fluorescence derivatization with 4-nitro-7-piperazino-2,1,3-benzoxadiazole (NBD–PZ). A high-performance liquid chromatographic separation of the β-HMA derivative was achieved using an octadecyl silica column in gradient elution. A wide dynamic range of β-HMA was tested and a calibration curve was constructed with accuracy of 90% and variability of less than 10%. The limits of detection and quantification obtained were 2 and 5 μM, respectively. The developed method was applied to detect endotoxins in dialysis water by alkaline hydrolysis of LPS using NaOH (0.25 M) at 60 °C for 2 h. After hydrolysis, free acid was detected as its NBD–PZ derivative using high-performance liquid chromatography/mass spectrometry (HPLC/MS). Good recovery rates ranging from 98 to 105% were obtained for β-HMA in dialysis water.

Lipopolysaccharides (LPSs,[1] also known as bacterial endotoxins or lipoglycans) are the main constituents of the outer membrane of gram-negative bacteria. LPS acts as an extremely strong stimulator of innate immunity in diverse eukaryotic species [1]. As depicted in Fig. 1, LPS is composed of three distinct domains: a glycolipid part named lipid A, a polysaccharide core, and an O-specific chain, consisting of 20 to 40 repeating units, which generally may contain up to eight different sugars. Although LPS can have beneficial effects at low concentrations (i.e., stimulating the immune response), it is toxic at higher concentrations and can lead to septic shock and even death [2,3]. Lipid A moiety of endotoxins is responsible for the toxic effects of LPS such as fever, tissue necrosis, and activation of the complement system. The structure of the lipid A portion is fairly well conserved, but the nature (length and chemical composition) of the polysaccharide side chain varies among genera, species, and even strains of gram-negative bacteria [4,5]. The endotoxic activity of LPS resides in the lipid A domain given that polysaccharide-deprived free lipid A appears to exhibit similar endotoxic activities as intact LPS [6,7]. Because lipid A is embedded in the outer membrane of bacterial cells, it probably exerts toxic effects only when released from multiplying cells in a soluble form or when the bacteria are lysed [8]. β-Hydroxyl acids, especially β-hydroxymyristic acid (β-HMA) [9,10] and β-hydroxylauric acid [11], constitute an essential part of lipid A and, therefore, have been used as chemical markers for quantification of lipid A. Lipid A in *Escherichia coli* is composed of β-1,6-linked d-glucosamine disaccharide units, 1,4-interlinked by pyrophosphate bridges. Amino groups are substituted with β-HMA; three hydroxyl groups in each disaccharide unit are linked with lauric, myristic, and palmitic acids, whereas one is joined to a 2-keto-3-deoxyoctanoate moiety [12,13].

During recent times, bacterial and endotoxin contamination of dialysis water have been a major problem. Dialysis patients are typically exposed to 90 to 120 L of dialysis fluid per treatment, which equates to an annual exposure of 20,000 to 30,000 L [14,15]. With constant exposure to large amounts of fluid, the opportunity for a dialysis patient to experience an inflammatory or pyrogenic reaction due to contamination within the dialysis fluid is increased. The metabolic, immunologic, and phlogosis effects of the release of interleukin-1 and tumor necrosis factor in dialysis water have been reported by different authors [16,17].

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[1] *Abbreviations used:* LPS, lipopolysaccharide; β-HMA, β-hydroxymyristic acid; LAL, limulus amebocyte lysate; UV, ultraviolet; HPLC, high-performance liquid chromatography; MS, mass spectrometry; NBD–PZ, 4-nitro-7-piperazino-2,1,3-benzoxadiazole; ACN, acetonitrile; TPP, triphenylphosphine; DPDS, 2,2′-dipyridil disulfide; DMF, dimethylformamide; TFA, trifluoroacetic acid; UPLC, ultra-performance liquid chromatography; CV, coefficient of variation; SD, standard deviation.

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For hemodialysis, fluids that are used for treatments do not need to be sterile; however, the lower the microbial concentration, the lower the risk of patient reaction. Because of this risk, regional regulatory boards have implemented limits to the total microbial count that can be present in fluids that are to be used in dialysis treatments. However, even if water treatment systems are in place, contamination is still a possibility and a risk. Dialysis fluid used for clinical treatments may become contaminated from the source water, the dialysate concentrate, or the water distribution system. Due to the ubiquitous nature of biofilm and its propensity to generate endotoxin, this problem affects not only hemodialysis but all extracorporeal therapies [18]. Hence, constant monitoring of endotoxin concentration is of great importance in dialysis water.

So far, the detection of LPS in clinical samples relies mainly on limulus amebocyte lysate (LAL) assay, which is similar to most enzymatic tests and very sensitive but is highly temperature and pH dependent. Some carbohydrate derivatives, such as glucans, may also give rise to false-positive results in LAL assay [19–21]. Thus, the development of enzyme-free LPS analysis methods becomes a focus of current research, and colorimetric, fluorescent, or electrochemical LPS chemosensors are intensively pursued by virtue of their high sensitivity and selectivity as well as their extraordinary simplicity in both detection agents and instrumentation [22–28].

A different approach to quantify endotoxins is represented by chemical analysis, which is based on the release and detection of β-HMA and, therefore, can be a chemical marker for LPS detection. β-HMA is the most abundant saturated fatty acid in the lipid A complex in most toxic gram-negative bacteria, constituting two residues per disaccharide unit [29]. Therefore, determination of β-HMA may be related to the endotoxin content of many toxic gram-negative bacteria. In such ways, gas chromatography coupled to mass spectrometry (GC–MS) and high-performance liquid chromatography coupled to a fluorescence detector, ultraviolet/visible (UV/VIS) spectrophotometry, or a triple quadrupole mass spectrometer (HPLC–MS/MS) are the analytical methods commonly used for this purpose [29–33]. Commonly, this analysis step is preceded by a derivatization step of hydrolyzed long-chain fatty acids (by silylation or conversion into methyl esters) [34].

The ultimate purpose of our current research dealt with the development of an on-line analyzer of endotoxins that could circumvent some drawbacks of the LAL-based methods and/or prevent the use of non-commercially available and synthetically expensive molecules and avoid multistage steps of endotoxin determination. The work presented here aimed to validate the use of 4-nitro-7-piperazino-2,1,3-benzoxadiazole (NBD–PZ) as a chemical derivatizing agent for quantitation of β-HMA as an LPS chemical marker in gram-negative bacterial strains such as *E. coli*. To verify the applicability of the developed method, we tested our alternative protocol with dialysis water samples containing a known amount of LPS, and HPLC–MS was used as the analytical method to optimize the derivatization reactions. Thus, the current work was guided not by seeking the best sensitivity but rather by seeking the best selectivity for β-HMA detection.

Materials and methods

Chemicals and reagents

Control Standard Endotoxin (0.5 μg/vial) was purchased from Cape Cod (East Falmouth, MA, USA). It is a purified extract of *E. coli* O113:H10, the same strain used for the U.S. Pharmacopeia and the European Pharmacopeia reference standard endotoxin. β-HMA and HPLC-grade acetonitrile (ACN) were purchased from Sigma–Aldrich (Illkirch, France). The fluorescent amine NBD–PZ, triphenylphosphine (TPP), and 2,2′-dipyridil disulfide (DPDS) were obtained from Tokyo Kasei Chemicals (Tokyo, Japan). Dimethylformamide (DMF) was obtained from Prolabo (Saint Herblain, France), and trifluoroacetic acid (TFA) was obtained from Merck (Darmstadt, Germany). Purified Milli-Q water was used for reagent preparations, whereas distilled water from a Distinction D4000 water still (Bibby Scientific, Roissy, France) was used to dissolve endotoxins. The derivatized samples were poured into 2-ml glass vials and injected into the HPLC system.

Safety note

Endotoxins are allergens and, hence, should be handled with extreme care. An organic vapor dust respirator mask (Sigma–Aldrich), nylon gloves, and safety goggles were used throughout the experiments.

Sample and reagent preparation

Stock β-HMA solution (5 mM) was prepared in ACN and further diluted in water before the experiments. NBD–PZ solutions were

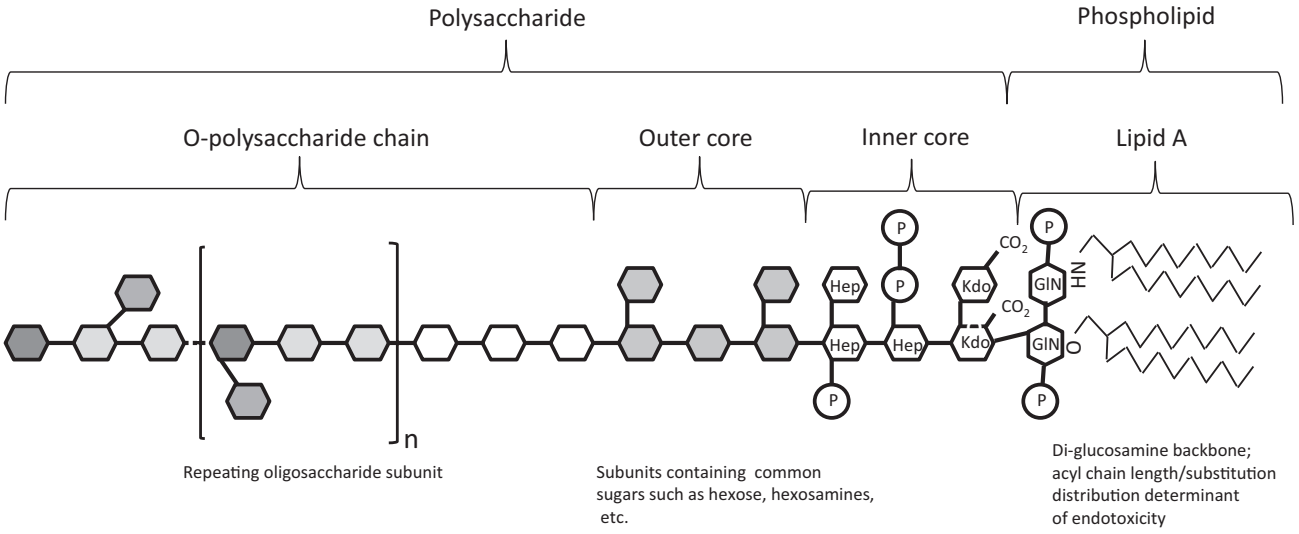

Fig.1. Schematic representation of lipopolysaccharide structural units. Hep, heptose units; P, phosphate units; Kdo, 3-deoxy-D-manno-2-octulosonic acid; GlN, glucosamine units.