A Quantitative Microanalysis of Bacterial Endotoxin Using \([^{3}\text{H}]\)-Labeled L-Glycero-D-mannoheptitol as a Marker

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Abstract: Quantitative microanalysis of bacterial endotoxin was performed using \([^{3}\text{H}]\)-labeled L-glycero-D-mannoheptitol (LD-Heptitol) as a marker. Several different amounts of authentic L-glycero-D-mannoheptose (LD-Heptose) were reduced with 20 μg of cold NaBH₄ containing 2 μg of NaB³H₄ (40 Ci/ mmol) in 20 μl of 1 mM NaOH at 4 C for 48 hr. The product, \([^{3}\text{H}]\)-labeled LD-Heptitol, has high specific activity, and was purified by HPLC and detected using a liquid-scintillation counter. As little as 50 pg of LD-Heptose was detectable, and the radioactivity increased dose-dependently in the 100 pg to 80 ng range tested. More than 2 ng of Salmonella abortus equi endotoxin could be accurately determined by this method. It is possible to detect 50 pg of endotoxin by this method, if 100% hot material (NaB³H₄) is used for \([^{3}\text{H}]\)-labeling.

Key words: Endotoxin, Lipopolysaccharide, L-Glycero-D-mannoheptose, \([^{3}\text{H}]\)-Label

As the result of accumulated findings in clinical and experimental studies (1, 9), endotoxin, which is chemically a lipopolysaccharide (10) located in the cell surface of Gram-negative bacteria, is thought to be closely related to death or shock in severe septic digestive diseases.

Limulus amebocyte lysate (LAL) assay (6) is highly sensitive to endotoxin, and is currently the common method for detecting and quantifying endotoxin. The LAL assay still has several drawbacks, however, when used to measure circulating endotoxin, of which the lipid A moiety is enzymatically degraded (5). The reaction of LAL reagent is strongly affected by the chemical and physicochemical structure of the endotoxin and modified drastically by the presence of inhibitors and activators (7, 12). Considering these points, the determination of endotoxin by LAL assay does not always reflect the real amounts of endotoxin.

To solve these problems, certain chemical methods have been developed for direct detection of endotoxin in serum or aqueous solution (2, 8, 13), all of which use 3-hydroxy tetradecanoic acid (3-OH C₁₄ : 0) in the lipid A portion as a marker. We now describe a highly sensitive method for measuring endotoxin in aqueous solutions by \([^{3}\text{H}]\)-labeling L-glycero-D-mannoheptose (LD-Heptose) which widely exists in the core part of endotoxin, as a tracer.

The endotoxin of Salmonella minnesota Rd₁P⁻SF1121 mutant provided by Dr. G. Schmidt, Forschungsinstitut Borstel (Germany), was prepared according to the method of (3, 4). S. abortus equi endotoxin was provided by Dr. C. Galanos, Max-Planck Institute, Freiburg (Germany).

Purified LD-Heptose (20.3 mg) was obtained from the hydrolysates [2 M trifluoroacetic acid (TFA), 100 C, 3 hr] of S. minnesota Rd₁P⁻ mutant endotoxin (170 mg) by HPLC using a Sugar Pak-Pb column (7.8 × 300 mm, Waters) with the mobile phase of distilled water at 80 C at 0.6 ml/min. A portion of the LD-Heptose (10.3 mg) was reduced with NaBH₄, purified by the same HPLC system (1 ml/min), and the L-glycero-D-mannoheptitol (LD-Heptitol) obtained (9.8 mg) was used as an authentic material. \([^{3}\text{H}]\)-Labeling of LD-Heptose was performed with NaBH₄/NaB³H₄ (40 Ci/mmol, 40 Ci/mmol, 3-OH C₁₄ : 0, 3-hydroxy tetradecanoic acid; Rt, retention time; TFA, trifluoroacetic acid; TLC, thin-layer chromatography.

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American Radiolabelled Chemicals) (9:1, w/w), and the mixture was acidified with acetic acid (50 μl) and evaporated under N₂ stream. The residue was codistilled two times with acetic acid and additional six times with methanol containing 1% acetic acid (50μl). The residue was then purified by repeated HPLC on AsahiPak GS-310H (7.6×250 mm, Asahi Chemical Industry) using CH₃CN/H₂O (9:1, v/v, 1 ml/min) and Sugar Pak-Pb columns, and the radioactivity of the LD-Heptitol fraction was measured using a liquid scintillation system LSC-700 (Aloka) in 5 ml of Aquasol-2 (NEN Research Products).

Thin-layer chromatography (TLC) analysis was performed on a silica gel 60 plate (Merck) with 2-propanol/1M ammonia/water (6:2:1, v/v) as an irrigant. Gas-liquid chromatography (GLC) and gas-liquid chromatography/mass spectroscopy (GC-MS) was carried out as described in the previous report (4). Pyridylamination of sugars and the separation by HPLC was carried out according to (11).

Purified LD-Heptose produced a single spot (Rf = 0.38) in TLC. The molecular mass of the [1-²H]-alditol acetate derivative [retention time (Rt) = 27.2 min] was determined to be 507 Da [m/z 508 (M+H)+ and m/z 525 (M+NH₄)+] by chemical-ionization mass spectrometry, The characteristic fragment ions of the derivative at m/z 434 and 435 such as C1-C6 and C2-C7 fragments were also observed in the electron-impact mass spectrum, confirming the expected structure. HPLC using Sugar Pak-Pb and AsahiPak GS-310H columns yielded good separation of LD-Heptitol (Rt = 21.7 and 42.0 min, respectively) from the most of the alditols of following sugar standards tested: ribose (13.9 and 20.9 min), arabinose (17.1 and 21.7 min), mannose (17.2 and 30.0 min), D-glycero-D-manno-heptose (18.1 and 37.4 min), D-mannoheptulose (18.3 and 21.6, and 38.9 and 44.1 min) which produces D-glycero-D-galacto- and D-glycero-D-talo-heptitols by the reduction, rhamnose (19.5 and 17.9 min), xylolose (21.4 and 20.7 min), galactose (21.6 and 31.0 min), fucose (21.8 and 18.1 min), glucose (23.3 and 28.3 min) and D-glycero-D-gulo-heptose (27.1 and 36.5 min).

Authentic LD-Heptose (20 ng) was reduced with 0.2, 2, 20 and 200 μg of NaBH₄ at 4 C for 48 hr in 20 μl of [A] 1 mM NaOH, [B] 1 mM NaOH/methanol (1:9, v/v), [C] 1 mM NaOH/ethanol (1:9, v/v) and [D] 1 mM NaOH/2-propanol (1:9, v/v). After decomposition of excess NaBH₄, the remaining LD-Heptose was pyridylaminated and quantified by HPLC. As the result, LD-Heptose was completely reduced with 20 μg of NaBH₄ in solvent systems A and C. In all solvent systems, no reduction or incomplete reduction was observed when less than 2 μg of NaBH₄ was used. Taking these findings into consideration, 20 μg of NaBH₄ in solvent system A was selected as the conditions for labeling minute amounts of LD-Heptose.

Various amounts of authentic LD-Heptose were [³H]-labeled under the conditions established above. The residue was applied to HPLC on an AsahiPak GS-310H column in the presence of 100 μg of cold LD-Heptitol in order to make detection by refractive index monitor possible and keep the loss of hot material to a minimum level. The LD-Heptitol fraction was collected, repurified with the same HPLC system, and further purified twice by HPLC on a Sugar Pak-Pb column followed by measuring radioactivity of the LD-Heptitol fraction. Total recovery of [³H]-LD-Heptitol was more than 90% after the entire procedure. Figure 1 shows the elution profile of [³H]-LD-Heptitol at the final HPLC. The radioactivity increased dose-dependently in the 100 pg to 80 ng range of LD-Heptose tested and as little as 50 pg of LD-Heptose was detectable in this system. The radioactivity incorporated into 100 pg of LD-Heptose was approximately 1,480 cpm, including the background (675.5 cpm). The analytical error at the dose was ±10% at the...
Maximal release of LD-Heptose from S. abortus equi endotoxin was observed after 8 hr hydrolysis (2 M TFA, 100°C) of the endotoxin. The LD-Heptose liberated was [3H]-labeled, purified on HPLC, and its radioactivity was measured. A linear relationship was obtained between the radioactivity and increasing amounts of endotoxin in the 2 ng to 80 ng range tested (Fig. 2). The minimum detectable amount of endotoxin with this system was 500 pg.

Fig. 2. LD-Heptose content of S. abortus equi endotoxin. LD-Heptose liberated from various amounts of the endotoxin by acid-hydrolysis was [3H]-labeled, and was purified by repeated HPLC in the presence of cold LD-Heptitol (100 μg). Radioactivity of LD-Heptitol fraction was measured using a liquid scintillation counter, and the amount of LD-Heptose was calculated from standard curve.

References


