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methods

Quantitative lipopolysaccharide analysis using HPLC/MS/MS and its combination with the limulus amebocyte lysate assay[S]

Jean-Paul Pais de Barros,[1,*,†,§] Thomas Gautier,[*,†,§] Wahib Sali,[*,†,§] Christophe Adrie,[**] Hélène Choubley,[§] Emilie Charron,[§] Caroline Lalande,[§] Naig Le Guern,[*,†,§] Valérie Deckert,[*,†,§] Mehran Monchi,[††] Jean-Pierre Quenot,[*,†,§,§§] and Laurent Lagrost[1,*,†,§,*]**

INSERM, LNC UMR866,* F-21000 Dijon, France; LNC UMR866,† University Bourgogne Franche-Comté, F-21000 Dijon, France; LipSTIC LabEx,§ Fondation de Coopération Scientifique Bourgogne-Franche Comté, F-21000 Dijon, France; Physiology Department,** Cochin Hospital, Paris University, Paris, France; Intensive Care Unit,†† Melun General Hospital, Melun, France; and Intensive Care Unit§§ and Clinical Research Department,*** University Hospital of Dijon, F-21000 Dijon, France

Abstract Quantitation of plasma lipopolysaccharides (LPSs) might be used to document Gram-negative bacterial infection. In the present work, LPS-derived 3-hydroxymyristate was extracted from plasma samples with an organic solvent, separated by reversed phase HPLC, and quantitated by MS/MS. This mass assay was combined with the limulus amebocyte lysate (LAL) bioassay to monitor neutralization of LPS activity in biological samples. The described HPLC/MS/MS method is a reliable, practical, accurate, and sensitive tool to quantitate LPS. The combination of the LAL and HPLC/MS/MS analyses provided new evidence for the intrinsic capacity of plasma lipoproteins and phospholipid transfer protein to neutralize the activity of LPS. In a subset of patients with systemic inflammatory response syndrome, with documented infection but with a negative plasma LAL test, significant amounts of LPS were measured by the HPLC/MS/MS method. Patients with the highest plasma LPS concentration were more severely ill. HPLC/MS/MS is a relevant method to quantitate endotoxin in a sample, to assess the efficacy of LPS neutralization, and to evaluate the proinflammatory potential of LPS in vivo.—Pais de Barros, J-P., T. Gautier, W. Sali, C. Adrie, H. Choubley, E. Charron, C. Lalande, N. Le Guern, V. Deckert, M. Monchi, J-P. Quenot, and L. Lagrost. **Quantitative lipopolysaccharide analysis using HPLC/MS/MS and its combination with the limulus amebocyte lysate assay. *J. Lipid Res.* 2015.** 56: **1363–1369.**

Supplementary key words inflammation • lipoprotein • lipid transfer protein • systemic inflammatory response syndrome • sepsis • human • mouse • diagnostic tool • mass spectrometry • liquid chromatography tandem mass spectrometry

Systemic inflammatory response syndrome (SIRS) is defined by a cluster of clinical signs that include tachycardia, leukocytosis, tachypnoea, and pyrexia (1). Although SIRS is a major consequence of sepsis (i.e., a deleterious, nonresolving inflammatory response to infection that can lead to multiple organ dysfunction and septic shock), it can be caused by many pathological events that are not associated with the bacterial infection (1). It is of major importance to distinguish between noninfectious SIRS, sepsis, and septic shock in critically ill patients because these groups of patients are markedly different in terms of care and clinical outcome. Positive microbiological identification tests [using conventional microbiological tests or bacterial DNA detection (2) as well as a number of inflammatory and infectious biomarkers, including cytokines, procalcitonin, immune cell markers or a combination of these (3)] have been proposed, but at this stage, none has proved to be reliable enough to ascertain the occurrence and extent of infection in high-risk patients with SIRS. Interestingly, and as documented further by our group in the prospective multicenter cohort EPISS study (4), Gram-negative bacilli are the most frequently identified pathogens in patients with septic shock. In this context, the direct quantitation of the culprit component of the Gram-negative bacterium [i.e., lipopolysaccharide (LPS), which triggers SIRS by interacting with the CD14/Toll-like receptor 4 /myeloid differentiation factor 2 receptor complex at the surface of leukocytes (1)], may be of high biological and clinical value.

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Abbreviations: 3HM, 3-hydroxymyristic acid or 3-hydroxymyristate; LAL, limulus amebocyte lysate; LOD, limit of detection; LOQ, limit of quantification; LPS, lipopolysaccharide; PLTP, phospholipid transfer protein; SIRS, systemic inflammatory response syndrome; SOFA, sequential organ failure assessment.

[1]To whom correspondence should be addressed.
e-mail: laurent.lagrost@u-bourgogne.fr (L.L.);
jppais@u-bourgogne.fr (J-P.P.d.B.)

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Because up to 100,000 molecules of LPS are known to locate at the surface of one single Gram-negative bacterium (1), LPS should provide an optimal and amplified signal to document bacterial infection in vivo.

Even though plasma LPS concentrations might be particularly relevant to document Gram-negative bacterial infection, it is still a major challenge to quantitate LPS in body fluids and tissues. Previously described assays include biological activity assays [such as limulus amebocyte lysate (LAL) and LAL-like activity assays], fluorescence assays (such as EndoLISA, Pyrogene, or EndoZyme), enzymatic tests (such as HEK-Blue LPS detection kit), and mass assays [such as 3-hydroxymyristate (3HM) quantitation by GC/MS] (5–8). These methods show some limitations. For instance, bioassays such as the endotoxin activity assay or LAL are restricted to the active LPS fraction and may not reflect total amounts of LPS in biological samples. They may thus produce false positive or false negative results (9). Quantitation of 3HM by GC/MS or GC/MS/MS corresponds to the direct measurement of LPS mass (8, 10, 11), but the detection of small amounts of 3HM in complex media is restricted by the limited amounts of lipid-rich samples that can be loaded on GC columns.

In the present study, a new HPLC/MS/MS method was set up for the sensitive, accurate, and direct quantitation of total amounts of LPS in plasma. This new method was compared with GC/MS and LAL assays, which were used in the present study as reference methods. Finally, the HPLC/MS/MS method was combined with the LAL assay in an attempt to monitor neutralization of LPS activity in biological media.

MATERIALS AND METHODS

Animals

Wild-type, C57BL6/J mice (3 months old) were provided by Charles River Laboratories International (L'Arbresle, France). Phospholipid transfer protein (PLTP)-deficient ($PLTP^{-/-}$) mice, generated by Dr. Jiang and colleagues (12), were on a homogeneous C57BL6/J background for at least eight generations. The mice were fed a standard chow diet (A03 diet; Safe, Augy, France) and had free access to water and food. All experiments involving animals were performed in accordance with the institutional guidelines and approved by the University of Burgundy's Ethics Committee on the Use of Laboratory Animals (protocol number 2511).

Reagents

LPS from *Escherichia coli*, serotype O55:B5, was purchased from Sigma (Sigma Chemicals). Intralipid was purchased from Fresenius Kabi, France. Chemical reagents used for chromatography were of the highest quality grade and purchased from Fisher Scientific. Internal standards were purchased from Matreya (Biovalley, France)

LPS injection and blood sampling

All materials were of pyrogen-free grade or made apyrogenic by overnight heating at 150°C, and all of the reagents used were of "endotoxin-free" grade. LPS from *E. coli*, serotype O55:B5 (Sigma Chemicals), was suspended in endotoxin-free, 0.15 M sodium chloride and vigorously mixed for 15 min before use. LPS was injected into the mice intraperitoneally (1.9 mg/kg body weight, single dose). Blood was then collected in heparin-containing tubes at the indicated times by retroorbital puncture. Plasma was obtained by blood centrifugation (10 min, 2,000 *g* at 4°C).

Human plasma samples

Plasma samples from 20 patients (11 males/9 females, 64.4 ± 17.9 years, body mass index 25.0 ± 8.2 kg/m^2) with infectious SIRS but presenting a negative LAL test had been collected in a previous study (2). Sepsis in all patients was defined according to the criteria of the panel of experts from the American College of Chest Physician/Society of Critical Care Medicine (13). Sites of infection were the lungs (12 cases), abdomen (4 cases), urinary tract (2 cases), and other (2 cases). Infection was documented by bacteriological analysis in 13 cases. Fifteen patients presented with cardiovascular disease, 4 with type 2 diabetes, 9 with respiratory disease, 3 with cancer, and 4 with impaired renal filtration. Fasting blood was collected in EDTA-containing tubes within the first 24 h of the sepsis episode, and plasma samples were kept at −80°C until analysis. The study protocol was approved by the institutional review board for human experimentation (Pitié-Salpêtrière's Ethics Committee), and written informed consent was obtained from the patients or their next of kin.

LAL assay

Biological activity of LPS was quantified by the end-point chromogenic LAL assay (QCL-1000 kit; Lonza, Walkersville, MD), which gives a yellow color when positive. Briefly, 50 µl of diluted plasma (1:1 to 1:1000 dilution in endotoxin-free water) was dispensed in each well of a 96-well plate. At the initial time point, 50 µl of the LAL reagent was added to each well. The plate was shaken and incubated at 37°C for 10 min. Then, 100 µl of chromogenic substrate warmed to 37°C was added to each well, and incubation was extended for an additional 6 min at 37°C. The reaction was stopped by adding 100 µl of a 25% solution of glacial acetic acid. Absorbance was measured at 405 nm on a spectrophotometer (Victor3, Perkin Elmer).

GC/MS analysis

LPS concentration was determined by direct quantitation of 3β-O-trimethylsilyl ether-tetradecanoic methyl ester by GC/MS. Briefly, 25 µl of plasma spiked with 50 ng of internal standard (3β-hydroxytridecanoic acid 10 mg/ml in ethanol) was hydrolyzed with 75 µl of NaCl 150 mM and 300 µl of HCl 8 M for 4 h at 90°C. Free fatty acids were then extracted with 600 µl of distilled water and 5 ml of hexane. After evaporation of the hexane phase, fatty acids were methylated with 250 µl of boron trifluoride/methanol (1:9) for 30 min at 60°C. The methylation solution was then evaporated under an N_2 stream. The dried extract was further incubated for 1 h at 80°C with 100 µl of *N,O*-bis(trimethylsilyl)trifluoroacetamide/trimethyl-chlorosilane (4:1) solution for derivatization. Silylating solution was then evaporated under an N_2 stream. Finally, 100 µl of hexane was added, and 1 µl of the mixture was injected in the split/splitless mode (split ratio 10:1) as previously described (14) on an Agilent GC 6890, MSD 5973 device.

HPLC/MS/MS

LPS concentration was determined by direct quantitation of 3-hydroxytetradecanoic acid (or 3HM) by LC/MS/MS. Briefly, 50 µl of plasma spiked with 50 ng of internal standard (3β-hydroxytridecanoic acid 10 mg/ml in ethanol) was hydrolyzed (total 3HM) or not (unesterified 3HM) with 75 µl of NaCl 150 mM and 300 µl of HCl 8 mol for 4 h at 90°C. Free fatty acids were then extracted with 600 µl of distilled water and 5 ml of hexane.

After vacuum evaporation of the hexane phase, fatty acids were dissolved in 100 µl of a 40% A/60% B eluent mixture (eluent A, ammonium acetate 5 mM pH 5.0; eluent B, acetonitrile/acetate ammonium 5 mM pH 7.3 96.7%/3.3%) (15). Fatty acid separation was performed in an Infinity 1200 HPLC binary system (Agilent) equipped with a Poroshell 120 EC C18 100 × 4.6 mm 2.7 µm column (Agilent) set at 30°C. The sample volume injected was 10 µl. A 7 min eluent gradient was established as follows: from 0 to 0.5 min, the flow was maintained constant at 1 ml/min of 80% B; then the proportion of B increased linearly up to 100% in 1 min; concomitantly the flow rate was decreased to 0.5 ml/min; these conditions were maintained constant for 1 min; then the flow rate was increased to 1 ml/min for an additional 2.5 min; finally, the column was reequilibrated with 80% B at 1 ml/min for 2.5 min. MS/MS detection was performed using a QQQ 6460 triple quadruple mass spectrometer (Agilent) equipped with a JetStream ESI source in the negative mode (gas temperature 300°C, gas flow 10 l/min, nebulizer 20 psi, sheath gas temperature 200°C, sheath gas flow 11 l/min, capillary 3,500 V). Nitrogen was used as the collision gas. The mass spectrometer was set up in the selected reaction monitoring (SRM) mode for the quantification of selected ions as follows: for 3-hydroxytetradecanoic acid, precursor ion 243.2 Da, product ion 59 Da, fragmentor 93 V, collision cell 9 eV; for 3-hydroxytridecanoic acid, precursor ion 229.2 Da, product ion 59 Da, fragmentor 110 V, collision cell 10 eV.

In human samples, LPS-derived/esterified 3HM was calculated as the difference between total 3HM (as assessed after HCl hydrolysis) and unesterified/free 3HM (as assessed without HCl hydrolysis).

Statistics

Linear regression analyses were performed using GraphPad Prism version 6.05 (GraphPad Software, San Diego, CA). For clinical data, values are presented as mean ± SD. Data were compared by the Mann-Whitney *U*-test. Differences with a *P* value below 0.05 were considered statistically significant.

RESULTS

Supplementary Fig. 1A, C shows the chromatograms of 3HM (extracted from plasma of mice intraperitoneally injected with LPS) and of hydroxytridecanoic acid (used as an internal standard) obtained with the HPLC/MS/MS method as described in Materials and Methods. As expected from molecular structures, 243.2 → 59 and 229.2 → 59 transitions were obtained by MS/MS for 3-hydroxytetradecanoic (retention time = 1.87 min) and 3-hydroxytridecanoic acid (retention time = 1.49 min), respectively (supplementary Fig. 1A, C). Extracted chromatograms of supplementary Fig. 1B, D correspond to the $[M\text{-}15]^+$ ion (*m/z* 301.2) of 3-O-trimethylsilyl ether-tridecanoic-methyl ester (retention time = 13.31 min), and the $[M\text{-}15]^+$ ion (*m/z* 315.2) of 3-OTMS-tetradecanoic-methyl ester (retention time = 15.27 min) as obtained by GC/MS, respectively. To assess the repeatability of the HPLC/MS/MS method, plasma samples were spiked with 3HM (final concentration, 50 ng/ml). The HPLC/MS/MS assay, conducted in triplicate in three independent experiments by three distinct operators, led to a coefficient of variation of 14% with a 92% recovery.

Plasma samples containing variable amounts of LPS were obtained from wild-type mice, which had been injected intraperitoneally with LPS from *E. coli* (O55:B5). The concentration of 3HM in the collected plasma samples (n = 71) was measured by the newly developed HPLC/MS/MS method and by GC/MS, which was used as the reference method. The linear regression analysis of concentrations obtained with the two methods gave a slope of 0.86 ± 0.01, an intercept of 4.97 ± 3.43, and an r^2 value of 0.9906 ($P < 0.0001$) (**Fig. 1A**). The limit of detection (LOD) and the limit of quantitation (LOQ) values for 3HM measurements in mouse plasma were 18 and 60 ng/ml with GC/MS and 12 and 40 ng/ml for HPLC/MS/MS, respectively (Fig. 1B). With 3HM concentrations below the LOQ values, a poorer but still significant correlation between the GC/MS and HPLC/MS/MS methods was observed (slope, 0.88 ± 0.16; intercept, 4.59 ± 2.99; $r^2 = 0.4587$, $P < 0.0001$) (Fig. 1B).

A significant but weaker correlation was observed when endotoxin values obtained over the entire range by using the newly developed HPLC/MS/MS method were compared with the classical LAL assay. Here, linear regression analysis gave a slope of 4.10E−5 ± 0.56E−5, an intercept

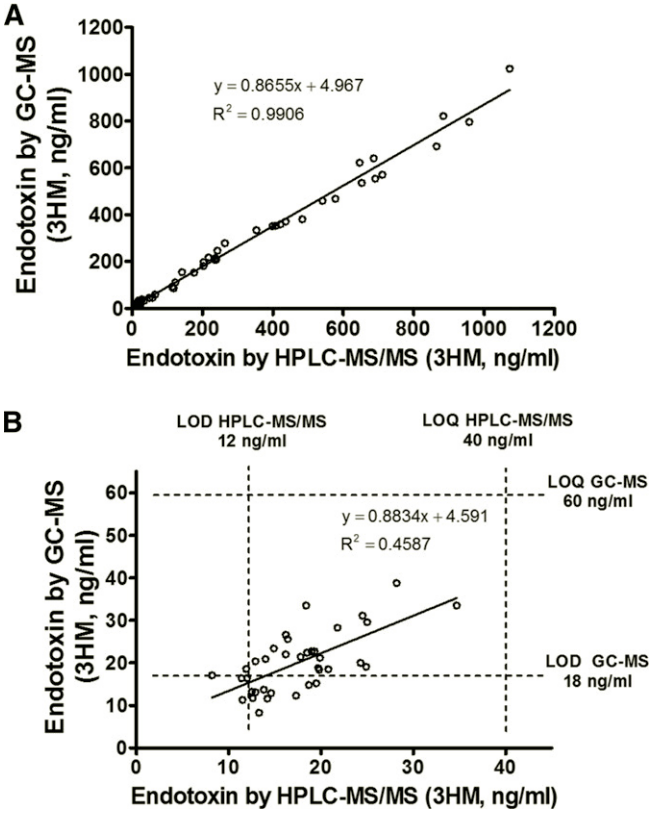

Fig. 1. Comparison of sensitivity and accuracy of HPLC/MS/MS and GC/MS techniques for LPS quantitation in plasma samples. Mouse plasma samples containing variable amounts of LPS were obtained after intraperitoneal injection of LPS from *E. coli* (O55:B5) and were subjected to HCl hydrolysis and lipid extraction as described in Materials and Methods. One aliquot of the resulting preparation was analyzed by HPLC/MS/MS, and another aliquot was analyzed by GC/MS for quantitation of 3HM and calculation of plasma LPS concentration (see Materials and Methods). A: Linear regression curve built by plotting LPS values determined by GC/MS against LPS values determined by HPLC/MS/MS. B: Linear regression curve as well as the determination of the LOD and LOQ of HPLC/MS/MS and GC/MS by using the lowest values (0–40 ng/ml range) of LPS levels in mouse plasma.

of 0.0418 ± 0.0017, and an r^2 value of 0.3886 ($P < 0.0001$) (**Fig. 2A**). In this case, however, there was no significant correlation between the two methods when LPS concentration values ranged between the LOD and LOQ (Fig. 2B).

As shown in supplementary Fig. 2, the HPLC/MS/MS method was relevant for the determination of 3HM levels in the lipid-rich, Intralipid medium, and consistent results were obtained with distinct LPS doses and after distinct incubation times at 37°C. Under the same experimental conditions, the chromogenic LAL assay was unable to quantitate LPS due to the high turbidity of the Intralipid mixture (not shown).

HPLC/MS/MS measurements of 3HM after spiking total plasma with known amounts of LPS were consistent with expected concentrations and were not affected by incubation at 37°C (**Fig. 3A**). In contrast to what was observed with HPLC/MS/MS, plasma samples from wild-type mice showed a potent and remarkable ability to turn down the LAL response in a time-dependent manner (Fig. 3B), and even with a high load of LPS, >85% of the initial LAL level could be blunted by 4 h of in vitro incubation of total plasma at 37°C, thus contrasting with the endurance of LPS measurements by the HPLC/MS/MS method. The ability of mouse plasma to mask the LAL response was further demonstrated in vivo. Whereas both LAL and HPLC/MS/MS assays peaked within a few hours following the initial intraperitoneal injection of LPS, the decay curve in plasma over a 24 h period was much steeper when LPS was assayed with the LAL method than with the HPLC/MS/MS assay (**Fig. 4**). This suggests that over time both neutralization and clearance of plasma LPS may have contributed to apparently lower values for the LAL assay at the 4, 6, and 24 h time points. To determine whether the combination of the biological LAL assay and the direct HPLC/MS/MS assay might be relevant in assessing LPS neutralizing activity, both assays were applied to plasma samples of wild-type mice (with naturally high PLTP activity and LPS detoxifying potential) and PLTP-deficient mice [with impaired ability to associate LPS with circulating lipoproteins, so as to detoxify LPS in the liver and to tone down the

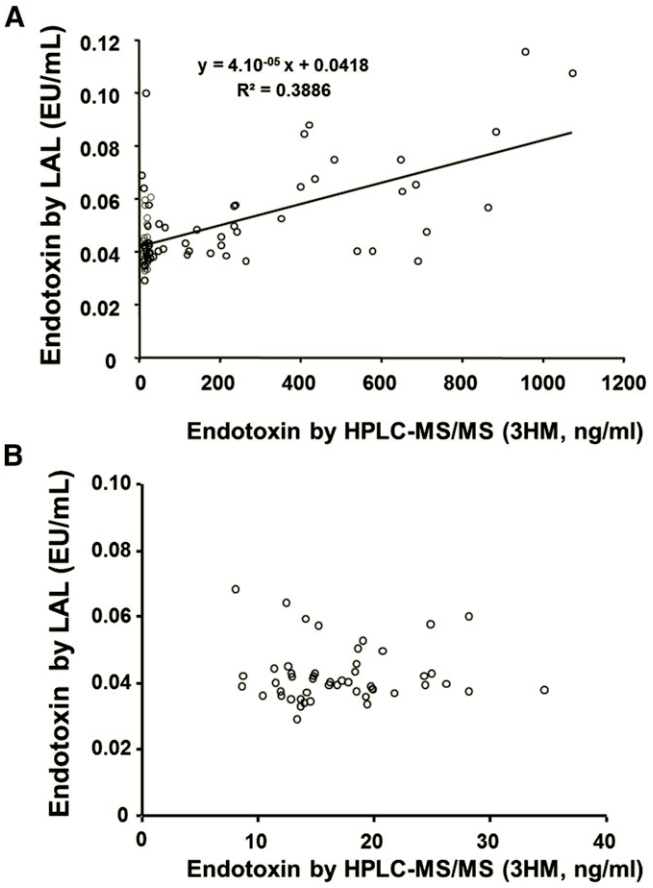

Fig. 2. Comparison of sensitivity and accuracy of HPLC/MS/MS and LAL techniques for LPS quantitation in plasma samples. Mouse plasma samples containing variable amounts of LPS were obtained after intraperitoneal injection of LPS from *E. coli* (O55:B5) and blood collection by retroorbital puncture. For each sample, one aliquot was directly analyzed by LAL assay, and another aliquot was subjected to HCl hydrolysis and lipid extraction prior to quantitation of 3HM by HPLC/MS/MS and calculation of plasma LPS concentration (see Materials and Methods). A: Linear regression curve built by plotting LPS values determined by LAL assay against LPS values determined by HPLC/MS/MS. B: The determination of the LOD and the LOQ of HPLC/MS/MS and LAL assay by using the lowest values (0–40 ng/ml range) of LPS levels in mouse plasma; no significant correlation between LAL and HPLC/MS/MS was observed in the low range of LPS levels (R^2 = 7E–5).

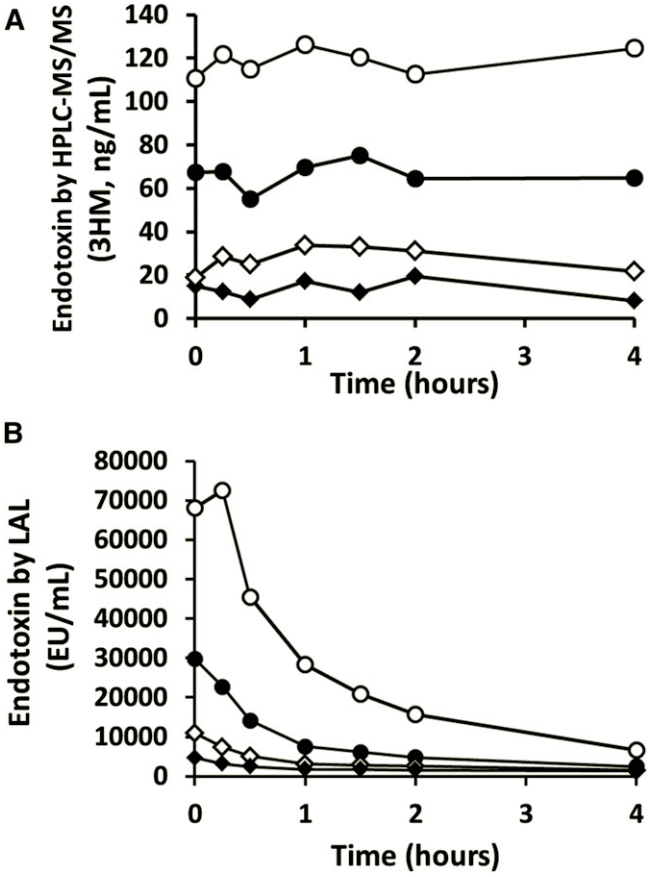

Fig. 3. Effect of incubation on the 3HM levels determined by HPLC/MS/MS in human plasma. Total human plasma was spiked with known amounts of LPS (closed diamonds, 300 ng/ml; open diamonds, 600 ng/ml; closed circles, 1,200 ng/ml; open circles, 2,400 ng/ml), and incubated at 37°C for 15 min, 30 min, 60 min, 90 min, 2 h, or 4 h. Incubated samples were analyzed by HPLC/MS/MS for quantitation of 3HM (A) and by LAL assay (B) as described in Materials and Methods.

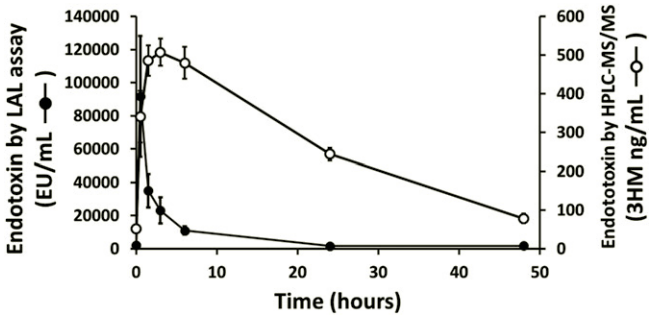

Fig. 4. Plasma kinetic curves of LPS levels determined by HPLC/MS/MS and LAL assay in wild-type mice. Wild-type C57BL/ mice (n = 6) were injected intraperitoneally with LPS from *E. coli* (O55:B5). Blood samples were drawn by retroorbital puncture before injection (time 0) and 0.5, 1, 3, 6, 24, and 48 h after injection, and plasma was obtained after centrifugation. For each plasma sample, one aliquot was analyzed by LAL assay (closed circles) and another by HPLC/MS/MS (open circles) for LPS quantitation as described in Materials and Methods. Values are expressed in ng/ml (for HPLC/MS/MS) or in endotoxin units (EU)/mL (for LAL) and are the means of 6 animals ± SEM.

inflammatory response (14)]. As shown in **Fig. 5**, and at the 0.5 and 1.5 h time points, the LAL to HPLC/MS/MS ratio values were significantly higher in PLTP-deficient than in wild-type mice.

Finally, and in order to bring insights into the pathophysiological and clinical relevance of the newly developed HPLC/MS/MS method, and to document its added value over the classical LAL assay, it was applied to the quantitation of LPS in total plasma of a subset of patients with SIRS, with documented infection but with a negative plasma LAL test. Remarkably, significant amounts of 3HM were measured in the plasma of SIRS patients, with total 3HM concentrations ranging between 11.2 and 50.2 ng/ml.

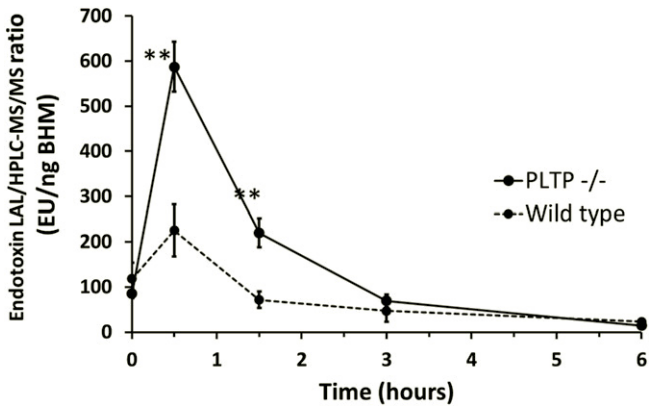

Fig. 5. Ratio of LPS activity to LPS mass concentration determined in wild-type and PLTP-deficient mice. Blood samples were drawn by retroorbital puncture before injection (time 0) and 0.5, 1.5, 3, and 6 h after injection of *E. coli* LPS (O55:B5, 1 mg/kg body weight). Plasma was obtained after centrifugation. LPS biological activity (LAL assay) to LPS mass concentration (direct 3-OH-myristate assay) ratios were determined over the 6 h period in wild-type (dotted line) and PLTP-deficient ($PLTP^{-/-}$) mice (full line). Values are mean ± SEM of 6 animals. ** $P < 0.01$, significantly different from wild-type mice.

Conducting or not conducting the initial HCl hydrolysis step made it possible to distinguish between unesterified 3HM and LPS-derived/esterified 3HM (i.e., total minus unesterified 3HM reflecting true LPS concentration). The distribution of patients on the two sides of the median LPS concentration of 11.4 ng/ml allowed us to distinguish between two groups with low/inframedian (LPS-derived 3HM, 8.8 ± 1.7 ng/ml) and high/supramedian (LPS-derived 3HM, 17.4 ± 7.1 ng/ml) LPS concentrations ($P < 0.0001$), but with no difference in unesterified 3HM concentrations (7.4 ± 3.2 vs. 10.4 ± 4.7, respectively; NS). Interestingly, patients in the high LPS subgroup showed significantly higher urea ($P < 0.005$), creatinine ($P < 0.05$), and renal sequential organ failure assessment (SOFA; $P < 0.05$) values and significantly lower pH values ($P < 0.05$) (**Table 1**).

DISCUSSION

In this study, we set up a new assay for the direct quantitation of LPS by using HPLC/MS/MS. The combination of the direct HPLC/MS/MS quantitative assay with the LAL biological activity assay is proposed as a relevant and practical approach to evaluate neutralization of LPS biological activity in a sample.

The present work relates to a method for quantifying 3HM [i.e., the most abundant hydroxylated fatty acid of the lipid A moiety of most LPS molecules (5, 16)], which was extracted with an organic solvent, separated by reversed phase HPLC and analyzed by MS/MS in a triple quadruple mass spectrometer. The HPLC/MS/MS method was shown to be reliable, practical, accurate, reproducible, and sensitive. Importantly, the intratest coefficient of variability for the LC/MS/MS assay was clearly below the poor values reported for LAL, which was thus considered a semiquantitative assay by others (17). In addition, the very good linear response for HPLC/MS/MS in both the low and high LPS range is compatible with the wide range of endotoxemia values that can be encountered in in vitro experiments, in animal studies, and in human populations. An additional advantage of the HPLC/MS/MS method lies in the fact that it was not affected by the lipid content of samples, even when LPS was added to lipid-rich Intralipid. This was an important point to confirm because of the lipidic nature of LPS, its propensity to associate with lipid-rich particles (18), and the confounding effect of elevated lipid levels in the LAL bioassay (18–20).

In earlier studies, the direct quantitative assay of 3HM was conducted by using GC/MS (8, 10, 11, 21, 22). In the present study, a highly significant correlation was observed between GC/MS and HPLC/MS/MS methods. LC requires more expensive equipment, but it avoids the methanolysis and derivatization step of GC, is less time consuming, and does not involve toxic reagents. Most importantly, the use of MS/MS in the SRM mode in our HPLC/MS/MS method allowed us to reach high specificity and low LOD and LOQ. This is particularly important in the context of LPS detection because even low-grade endotoxemia values are known to be sufficient to trigger an inflammatory response in vivo (23, 24).

TABLE 1. Biological and clinical parameters of subgroups of SIRS patients according to plasma LPS concentration

	LPS Below Median <11.4 ng/ml	LPS Above Median >11.4 ng/ml	*P*
N	10	10	
Total 3HM (ng/ml)	16.1 ± 4.1	27.8 ± 10.8	0.0067
Unesterified 3HM (ng/ml)	7.4 ± 3.2	10.4 ± 4.7	0.1419
LPS hydroxymyristate (ng/ml)	8.8 ± 1.7	17.4 ± 7.1	0.0001
Urea (mM)	6.8 ± 3.5	16.7 ± 7.4	0.0015
Creatinine (μM)	102.9 ± 56.1	167.1 ± 85.9	0.0273
pH	7.41 ± 0.07	7.28 ± 0.13	0.0124
Renal SOFA score	0.20 ± 0.42	1.30 ± 1.16	0.0233

LPS-derived/esterified 3HM was calculated as the difference between total 3HM (as assessed after HCl hydrolysis) and unesterified/free 3HM (as assessed without HCl hydrolysis). Values are presented as mean ± SD. Data means were compared by the Mann-Whitney *U*-test.

Following a single, intraperitoneal injection of LPS in wild-type mice, clear differences appeared over time between the LAL and the HPLC/MS/MS assays. Indeed, the biological activity of LPS as measured by the LAL assay returned to barely detectable levels 6 h after injection, while direct quantitation of 3HM by HPLC/MS/MS revealed that LPS actually remained at elevated levels in the plasma compartment of wild-type mice for much longer; high levels of LPS were still detectable even at the 24 h time point. Most importantly, quantitative analysis by HPLC/MS/MS revealed that maximal plasma levels of LPS were actually measured after 6 h, thus indicating that *1*) the bulk of circulating LPS was still present in the plasma compartment at this time, and *2*) in accordance with earlier reports (8, 25), the LAL assay can produce false negative results in native plasma. These findings are in agreement with earlier studies, which reported that in vitro incubation of plasma can mask LPS detection in a concentration-dependent manner (26–28). The combination of the LAL and HPLC/MS/MS analyses in the present study provided new evidence of the intrinsic capacity of plasma to neutralize the activity of LPS. Alteration of the LAL to HPLC/MS/MS ratio may to a large extent be due to the recognized ability of endogenous plasma lipoproteins to bind and neutralize LPS, because significantly lower values were calculated in wild-type mice than in PLTP-deficient mice, which are known to have a weaker ability to associate LPS with lipoproteins and to neutralize its biological activity (14). Here, rather than a surrogate for quantitation of total LPS amounts, the LAL assay was used as a direct in vivo evaluation of biologically active LPS. Finally, calculation of the LAL (activity) to HPLC/MS/MS (mass) LPS ratio is proposed as a relevant method to assess in vivo the efficacy of the initial step of LPS neutralization [known as the endotoxin-lipoprotein hypothesis (29)]. This step is followed by lipoprotein-mediated transport to the liver and ultimate detoxification in the bile (30, 31).

Even though the LAL assay has been used in most studies dealing with endotoxemia measurement, it has repeatedly been reported to be negative in some patients with sepsis (32, 33), and not to be clinically useful for detecting Gram-negative infections or predicting patient outcomes (16, 34, 35). Surprisingly, earlier studies reported that LAL positivity was observed in only 20% of patients presenting with sepsis at entry into an intensive care unit (16, 35). This indicates that the LAL assay cannot accurately identify patients with Gram-negative infection and endotoxemia. The present study brings direct evidence of the advantages of the HPLC/MS/MS method, in particular because it precludes at least some of the false negative results of LPS bioassays. In our study, conducting or not conducting the initial HCl hydrolysis step made it possible to distinguish between unesterified 3HM and LPS-derived/esterified 3HM (i.e., total minus unesterified 3HM reflecting true LPS concentration). Here, despite a negative LAL test, patients showed substantial LPS levels independently of free/background 3HM concentration values. Alternatively, the HPLC/MS/MS method can also apply to other hydroxylated fatty acid species (i.e., hydroxylated lauric acid) that are frequently present in LPS structures of some bacterial species (16). Going further, the HPLC/MS/MS method could be extended to a global lipidomic approach that monitors all hydroxylated fatty acyl chains.

Although the present study was not designed to address clinical outcomes, the subgroup of patients with supramedian plasma LPS levels showed higher urea, creatinine, and renal SOFA values and lower pH values than did patients with inframedian plasma LPS levels, thus indicating that they were more severely ill. These results are in agreement with earlier studies, which showed that endotoxemia was related to disease severity, the SOFA score, a higher plasma lactate concentration, lower systemic vascular resistance, depressed ejection fractions, a greater need for vasopressors, and a greater risk of dying (35–37). Thus, it can be anticipated that LPS measurement by HPLC/MS/MS could become a promising tool in the diagnosis, follow-up, and therapeutic management of patients with sepsis. It might also be relevant in investigating other pathophysiological states that may be associated with acute or low-grade endotoxemia (including cirrhosis, inflammatory bowel diseases, periodontitis, obesity, type 2 diabetes, and high fat feeding) (22, 38–41).

In conclusion, the combined results of the LAL and HPLC/MS/MS analyses bring to the fore a novel way to assess LPS neutralization capacity in vivo and to determine the proinflammatory potential of LPS molecules when present in various biological media. Whether there may be a place for routine HPLC/MS/MS measurement of endotoxemia in clinical practice and whether its combination with the LAL

assay is promising for the quantitation of inactive LPS and for monitoring the endotoxin neutralizing capacity of patients deserve further attention.

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