COMMUNICATION

Structural Characterization of a "Signature" Phosphatidylethanolamine as the Major 10-Hydroxy Stearic Acid-Containing Lipid of *Cryptosporidium parvum* Oocysts

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ABSTRACT: A 10-hydroxy stearic acid-containing lipid from *Cryptosporidium parvum* was purified by thin-layer chromatography and analyzed by infrared spectroscopy, fast-atom bombardment mass spectrometry, ^{1}H and ^{31}P nuclear magnetic resonance spectroscopy, and was identified as phosphatidylethanolamine.
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Detection and removal of potentially pathogenic and nonculturable microorganisms are major problems in the water industry. Particularly alarming are the protozoal cysts of *Giardia* and oocysts of *Cryptosporidium* which are nonculturable with standard bacterial techniques, and are responsible for diarrhea worldwide (1,2). *Cryptosporidium parvum* is an important and serious pathogen because it infects a wide range of mammalian hosts and patients with acquired immune deficiency syndromes (3). The current methods available to detect oocysts of *C. parvum* in water are currently unsatisfactory (4).

The chemical analysis of oocysts based on lipids may provide a useful method for the sensitive and rapid detection of *C. parvum* (White, D.C., Alugupalli, S., Schrum, D.P., Kelly, S.T., Sikka, M.K., Fayer, R., and Kaneshiro, E.S., unpublished data). The importance of this type of chemical analysis to compare the lipid compositions of the *C. parvum* and Madin-Darby bovine kidney cells has previously been reported (3). In our recent studies, we have shown that the lipid analysis could differentiate between *C. parvum* and *C. muris* (White, D.C., Alugupalli, S., Schrum, D.P., Kelly, S.T., Sikka, M.K., Fayer, R., and Kaneshiro, E.S., unpublished data). The present study was undertaken in order to chemically characterize the ester-linked native form of 10-OH 18:0 in *C. parvum*.

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Abbreviations: FAB, fast-atom bombardment; GC/MS, gas chromatography/mass spectrometry; NMR, nuclear magnetic resonance; PE, phosphatidylethanolamine; TLC, thin-layer chromatography; TMS, trimethyl silylation.

MATERIALS AND METHODS

Chemicals. All reagents were of analytical grade. *N,O*-Bis trimethylsilyl trifluoroacetamide, acetyl chloride, pyridine, and phospholipase A_2 (*Crotalus adamanteus* venom) were purchased from Sigma (St. Louis, MO). The standards 3-hydroxy tridecanoic acid and 6-hydroxy stearic acid were purchased from Matreya (Pleasant Gap, PA). The solvents hexane, chloroform, methanol, diethyl ether, and methylene chloride were also used.

Parasites. Oocysts of *C. parvum* used in this study were purified from calf feces as discussed previously (White, D.C., Alugupalli, S., Schrum, D.P., Kelly, S.T., Sikka, M.K., Fayer, R., and Kaneshiro, E.S., unpublished data).

Lipid extraction. Oocysts of *C. parvum* were extracted in a modified single-phase solvent system which included phosphate buffer (White, D.C., Alugupalli, S., Schrum, D.P., Kelly, S.T., Sikka, M.K., Fayer, R., and Kaneshiro, E.S., unpublished data; 5,6), at room temperature in chloroform/methanol/potassium phosphate buffer (50 mM, pH 7.4) (1:2:0.8, by vol) for 3 h. Chloroform and nanopure completely deionized, filtered water (1:1, vol/vol) were then added to cause a phase separation. The organic phase was then collected and dried under a stream of N_2 at 37°C.

Purification of lipids. The total lipid extract was dissolved in chloroform (~0.5 mL) and separated using a silicic acid column (10-cm column length, 0.5-cm inner diameter, 100–200 mesh silicic acid particle size) eluted with 5 mL of chloroform, 5 mL acetone, and 5 mL of methanol to elute neutral, glyco-, and phospholipids, respectively (7). Eluates were analyzed by silica-coated thin-layer chromatography (TLC) plates developed with chloroform/methanol/water (30:8:1, by vol). Sugar-containing compounds were visualized by spraying the plates with α-naphthol and heating at 110°C. Phospray reagent (Supelco, Bellefonte, PA) was used for revealing phosphorus-containing compounds, and ninhydrin reagent was used to reveal the presence of amino-containing species. Each individual lipid fraction was also ana-

lyzed for its fatty acid content. Final purification of the hydroxy fatty acid-containing lipid was achieved by reapplying the hydroxylated fatty acid-rich fractions on preparative TLC plates.

Selective phospholipase A_2 hydrolysis of phosphatidylethanolamine (PE) (8,9). Lyophilized phospholipase A_2 (250 units) was dissolved in 1 mL of 0.5 M Tris buffer with 0.4 M $CaCl_2$, at a pH of 7.5. A 300-µL aliquot of this solution was added to 0.4 mg of PE purified from *C. parvum* dissolved in 500 µL diethyl ether and shaken vigorously at 25°C for 20 min. The ether layer was removed, and the aqueous phase was washed with diethyl ether (2 × 1 mL). The ether washings were combined with the original ether layer, washed once with water (1 mL), collected, and dried under a stream of N_2 at 37°C. The fatty acids were subjected to acid methanolysis [1 M methanolic HCl (1 mL), 80°C, 30 min] and the hydroxy fatty acid methyl esters were separated, subjected to trimethyl silylation (TMS) and analyzed by gas chromatography/mass spectrometry (GC/MS) as previously described (10).

Other analytical techniques: (i) Characterization of the fatty acyl substituents. The acetone-eluted fraction from the silicic acid column was subjected to both mild and strong acid methanolysis as described previously (10). Fatty acid methyl esters were analyzed by GC/MS after the separation of hydroxy fatty acid methyl esters from nonhydroxylated compounds. The former class of fatty acid methyl esters was subjected to TMS derivatization prior to GC/MS analysis as described previously (White, D.C., Alugupalli, S., Schrum, D.P., Kelly, S.T., Sikka, M.K., Fayer, R., and Kaneshiro, E.S., unpublished data; 10).

(ii) Spectroscopy and spectrometry. Infrared spectra of samples were collected on a Bio-Rad FTS-60A Stepscan Interferometer (Bio-Rad Laboratories, Cambridge, MA) and measured as a thin film on NaCl plates.

^1H and ^{31}P nuclear magnetic resonance (NMR) spectra were obtained in $CDCl_3$ or $CDCl_3/CD_3OD$ (2:1, vol/vol) at 40°C on a modified NT360 NMR spectrometer (Nicolet, Madison, WI), operating at frequencies of 360.007 and 145.74 MHz, respectively.

Fast-atom bombardment (FAB) mass spectra were recorded with a ZAB-EQ (VG Analytical, Manchester, United Kingdom). Samples were dissolved in $CHCl_3$, and 1 µL was mixed on the probe tip with a matrix consisting of thioglycerol and 1% trifluoracetic acid. FAB was generated by an 8 keV xenon atom beam.

GC/MS was performed on a Trio-1 (VG Mass Lab) coupled to a Hewlett-Packard model 5890 gas chromatograph (Palo Alto, CA). Chromatographic separations were carried out on a fused-silica capillary column [30 m × 0.32 mm (inner diameter)] coated with cross-linked HP-1. Splitless injections were performed with a Hewlett-Packard model 7673 autosampler; the split valve was opened 1 min after injection. The carrier gas, helium, was used at an inlet pressure of 0.75 bar. The column temperature was programmed from 120 to 290°C, at 20°C/min; both the injector and the interface (between GC and MS) temperatures were kept at 280°C. The ionization was performed at 70 eV. The ion source temperature was 220°C in the electron impact mode. Hydroxy fatty acid methyl esters were identified and quantified as described previously (10).

RESULTS

Mass spectra. The electron impact mass spectrum of the TMS derivatized methyl ester ether (Me/TMS) of 10-OH 18:0 exhibited the characteristic fragments at *m/z* 215 (cleavage between C_9 and C_{10}), at *m/z* 273 (cleavage between C_{10} and C_{11}), and the molecular-specific ion at *m/z* 371 (M – loss of CH_3) (Fig. 1). Hydrolysis under both mild and strong conditions indicated that the 10-OH 18:0 is found mainly as ester-linked to the purified lipid.

Furthermore, the selective hydrolysis of PE purified from *C. parvum* with phospholipase A_2 indicated that 10-OH 18:O is located only on the *sn*-2 position as analyzed by GC/MS (data not shown).

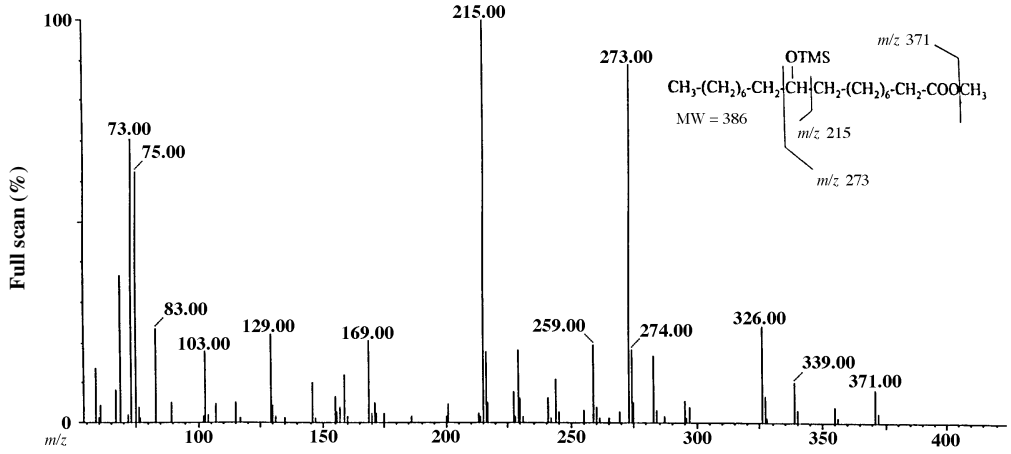

FIG. 1. Electron impact mass spectrum of the Me/trimethyl silylation (TMS)-derivatized 10-OH 18:0 found in *Cryptosporidium parvum*; MW, molecular weight.

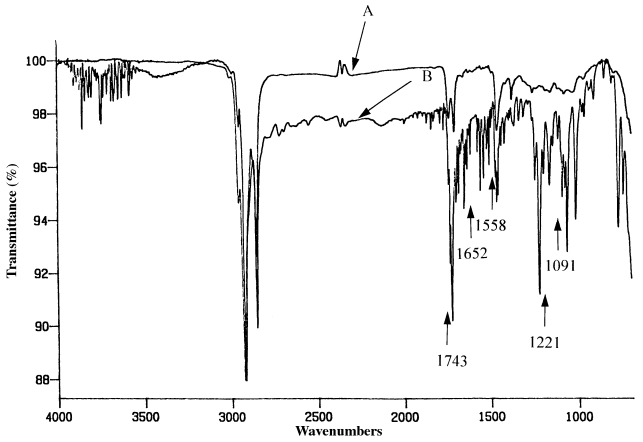

FIG. 2. Infrared spectrum of the purified phosphatidylethanolamine of *C. parvum* (marked A) and that of authentic phosphatidylethanolamine (PE) (marked B). The arrows indicate characteristic absorption bands typifying PE. See Figure 1 for abbreviation.

Fractionation of the total lipid extract by column chromatography followed by preparative TLC of the acetone-eluted fraction allowed the purification of the three lipids. The major lipid purified by preparative TLC was ninhydrin positive and phospray positive with the chromatographic mobility in one-dimension of authentic PE. This component lipid containing the ester-linked 10-OH 18:0 was also shown to be similar to authentic PE from its infrared, FAB/MS, ^{1}H NMR, and ^{31}P NMR spectra.

Figure 2 displays the infrared spectra of the major isolated lipid, which exhibited characteristic PE absorption bands at 1221 and 1091 cm^{-1} indicative for PO_2^-, 1652 and 1558 cm^{-1} indicative for NH_3^+ bands, 1743 cm^{-1} indicative of C=O bands, and 2850, 2920, and 2955 cm^{-1} characteristic of the aliphatic CH_3 and CH_2 stretching regions (between 2800 and 3000 cm^{-1}) (11). The positive FAB mass spectra of the native lipid (Fig. 3) showed the quasimolecular ion at *m/z* 664, confirming the PE structure and the presence of hydroxylated fatty acid substituents. The presence of ion at *m/z* 299 suggests that the lipid is acylated with hydroxylated stearic acid (12,13). Further confirmation of the lipid was also verified by ^{1}H and ^{31}P NMR to establish the configuration and the nature of the lipid (data not shown) (14).

DISCUSSION

The occurrence of 10-OH 18:0 in *Cryptosporidium* (White, D.C., Alugupalli, S., Schrum, D.P., Kelly, S.T., Sikka, M.K., Fayer, R., and Kaneshiro, E.S., unpublished data) led us to investigate the complex native form of this fatty acid in *C. parvum*. Comparision of the mild and strong methanolysis products has shown that 10-OH 18:0 occur in their native form mainly as an ester-linked lipid. Three lipids containing ester-linked 10-OH 18:0 were noted in the present study; however, the emphasis was placed mainly on the major lipid for structural characterization and molecular weight determinations. To the best of our knowledge, the present study is the first report of the presence of a 10-OH 18:0-containing lipid in *C. parvum*. The analysis reported herein shows that the extremely unusual fatty acid, 10-OH 18:0 which was previously reported in *Mycobacterium tuberculosis* lipids (15), is present in the PE of oocysts of *C. parvum* maintained under conditions where they retain infectivity for BALB/c neonatal mice.

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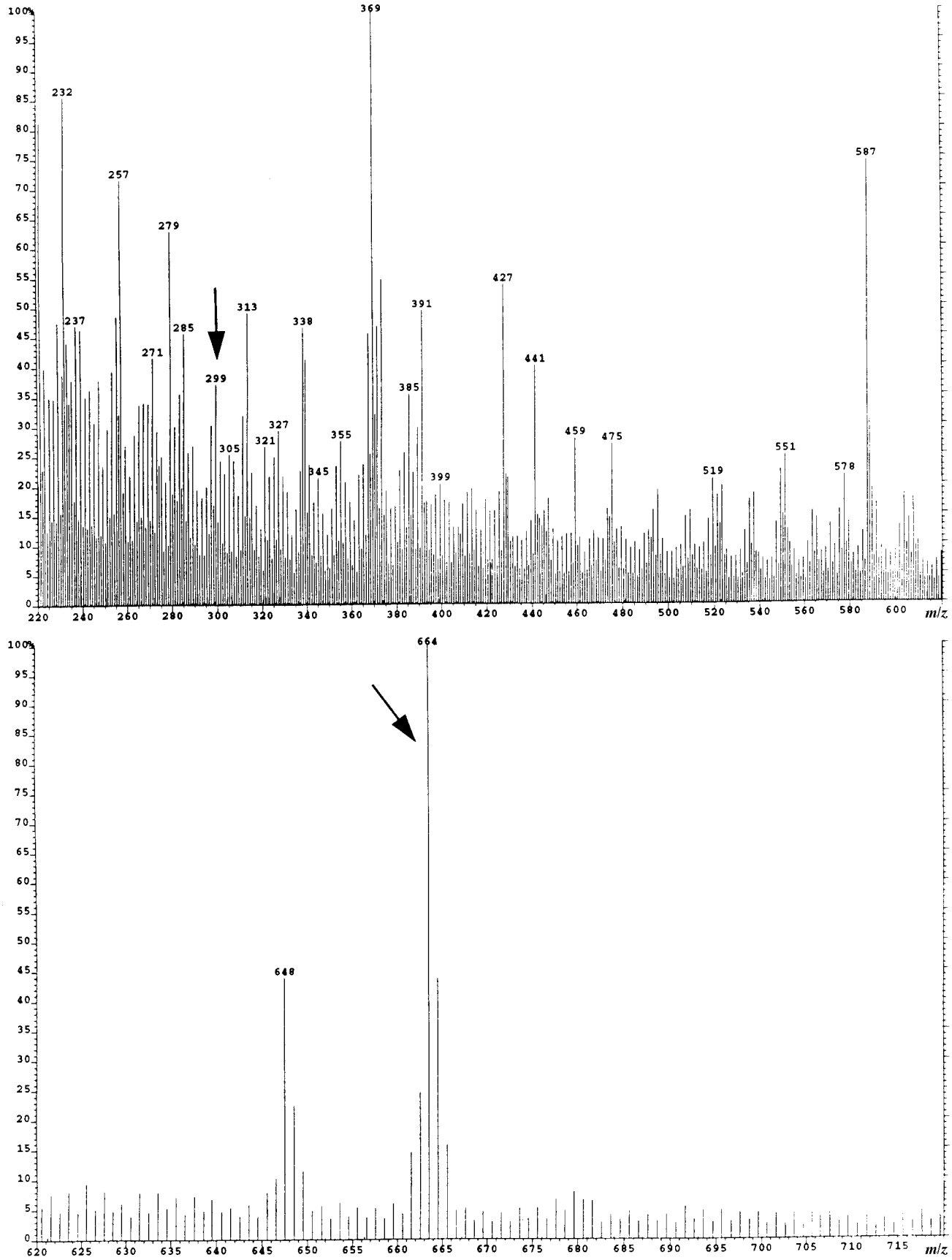

FIG. 3. Fast-atom bombardment-mass spectrum of the purified PE of *C. parvum*. Arrows point out molecular-ion peak ($[M + H]^+ = 664$) and peak corresponding to 10-OH 18:0. See Figures 1 and 2 for abbreviations.

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