The Signature 10-Hydroxy Stearic Acid Thought to Correlate with Infectivity in Oocysts of *Cryptosporidium* Species Is an Artifact

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ABSTRACT: Heating or freezing leads to loss in infectivity of oocysts of *Cryptosporidium parvum* toward neonatal BALB/c mice and is reflected in the profile of the polar lipid fatty acids. Upon loss of infectivity, the ratio of polar lipid to neutral lipid fatty acid decreased and the relative proportions of 18:1n-9 also decreased; proportions of 18:2n-6 and 20:5n-6 increased, whereas the proportions of 16:0 remained constant with freezing. During these investigations, a novel fatty acid, 10-OH 18:0, was discovered in the glycolipid fraction. The identification of a fatty acid unique to species of *Cryptosporidium* was thought to provide a specific biomarker for this organism. *Cryptosporidium* also demonstrated fluctuations in absolute quantities of 10-OH 18:0 with events that lead to loss of infectivity. This led to the presumed correlation of this biomarker with infectious *Cryptosporidium*. The 10-OH 18:0 was putatively localized at the *sn*-2 position of phosphatidylethanolamine. High-performance liquid chromatography/electrospray ionization mass spectrometry revealed that the 10-OH 18:0 existed principally in the free fatty acid form. Herein, we establish that the free fatty acid 10-OH 18:0 was, in actuality, an artifact of the procedures for sample preparation.

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A major problem in the potable water industry is the detection, identification, and determination of the presence of infectious protozoan parasites. *Cryptosporidium* is of special concern as it is widespread in surface water samples, has extreme resistance to disinfection by chlorine, completes its infectious cycle only in human or mammalian hosts, and is potentially fatal to immunocompromised individuals who become infected (1). Successful methods must be highly sensitive, as the presence of as few as 10–30 oocysts may initiate infections. The method must also be highly specific to *Cryptosporidium*, sufficiently rapid so as to be potentially useful to the water industry, and provide an indication of viability/infectivity. To date, existing methodologies for the detection of *C. parvum* oocysts in drinking water are not satisfactory (2). With the discovery that freezing *C. parvum* oocysts at –70°C, which renders them noninfectious to neonatal BALBC/c mice (3), induced shifts in the polar lipid/neutral lipid ratio, a shift in fatty acid composition, a decrease in cholesterol, and a loss in an unusual fatty acid 10-hydroxy stearic acid (10-OH 18:0) (4), a potentially quantitative biomarker was proposed. Identification of this fatty acid as unique to species of *Cryptosporidium* would provide a signature lipid biomarker (SLB) in complex environmental matrices such as water distribution system biofilms. For this reason, the focus of the assay was shifted to the highly unusual fatty acid, 10-OH 18:0. Previous investigators had shown that the traces of 10-OH 18:0 were localized at the *sn*-2 position of phosphatidylethanolamine (PE) (5). However, the combined efforts of parent/derived fragment ion mass spectrometry and high-performance liquid chromatography/electrospray ionization/mass spectrometry (HPLC/ESI/MS) by R.S. Burkhalter and C.A. Smith revealed that the 10-OH 18:0 existed in the free fatty acid form (6). Free fatty acids are extremely unusual in nature and the potential of 10-OH 18:0 as a unique "signature" was explored. Unfortunately, subsequent experimental evidence revealed the true origin of 10-OH 18:0. The 10-OH 18:0 was found to be an extractable component of the disposable rubber pipette bulbs used during purification and was not found as a lipid component of *Cryptosporidium* oocysts.

MATERIALS AND METHODS

Chemicals. All reagents were of analytical grade. The standards (3-hydroxy eicosanoic acid, 12-hydroxy stearic acid, and 6-hydroxy stearic acid) were purchased from Matreya (Pleasant Gap, PA). Chromatography-grade solvents of chloroform, methanol, acetone, and water were also used (4,5).

Rubber bulbs. Amber rubber bulbs (2-mL, catalog number 14065B; Fisher Scientific Co., Pittsburgh, PA) were utilized with glass pipettes to transfer solutions.

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Abbreviations: CID, collision-induced dissociation; FAB, fast ion bombardment; GC, gas chromatography; HPLC/ESI/MS, high-performance liquid chromatography/electrospray ionization/mass spectrometry; PE, phosphatidylethanolamine; 10-OH 18:0, 10-hydroxy stearic acid.

Parasites. Oocysts of *C. parvum* used in this study were purified from calf feces as previously described (4,5).

Lipid extraction. Membrane filter retentates or lyophilized sediments were extracted at room temperature and atmospheric pressure with the one-phase chloroform/methanol/phosphate buffer (50 mM, pH 7.4), 1:2:0.8 by vol, solvent system as previously described (7–9). All solvents were of gas chromatography grade and obtained from Baxter Scientific Products (McGaw Park, IL). All glassware was washed in a 10% (vol/vol) Micro cleaner solution (Baxter Diagnostics, Deerfield, IL), rinsed five times with tap water and then five times in deionized water. The glassware was then heated overnight in a muffle furnace at 450°C. Glass filter loaded samples were extracted in separatory funnels containing the chloroform/methanol/phosphate buffer, 1:2:0.8 single phase extractant 4 h before adding chloroform and deionized water (final solvent ratios, chloroform/methanol/ phosphate buffer/water; 1:1:0.4:0.5 by vol) to form two phases. 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 particle size). Each sample was loaded onto the column in a minimal volume of chloroform (100–200 μL) and then eluted in batches with 5 mL of chloroform, 5 mL acetone, and 5 mL of methanol so as to elute neutral, glyco-, and polar lipids, respectively (9). Prepacked silicic acid columns (Burdick and Jackson inert solid phase extraction system #7054G; Muskegon, MI) are also a commercial alternative. Bulk elution of the neutral lipids with chloroform was followed by recovery of the free fatty acids in acetone or glycolipid fraction. For each fraction, the solvent was removed under a stream of nitrogen. The lipids were stored at –20°C until analysis.

HPLC/ESI/MS. The acid 10-OH 18:0 migrates in reverse-phase high-performance liquid chromatography (C-18 Alltech Altima 5 μm particle size, 150 mm in length, 1 mm diameter column; Deerfield, IL) with a mobile phase consisting of methanol/ultrapure water/aqueous ammonia (100:6:1) at a flow rate of 50 μL/min with a retention time of 12–14 min typical of hydroxylated free fatty acids (3-OH 20:0). In contrast, PE elutes at 20–22 min under these conditions. Those components eluting with a retention volume typical of free fatty acids were nebulized into the ESI inlet of either the VG (Micromass Inc., Beverly, MA) Quattro II triple quadrupole mass spectrometer or the Micromass Platform II single quadrupole mass spectrometer. Analyses were performed in the negative mode of ionization so as to promote fragmentation resulting from cleavage of the two-acyl functionalities with charge retention on the free fatty acids formed through acyl cleavage. Initially, elevated cone voltages were utilized so as to promote fatty acyl cleavage from intact phospholipids. However, upon realizing that the biomarker of interest existed in the free fatty acid form, low orifice voltages were utilized so as to promote "soft" ionization. Tuning was performed such that maximum sensitivity was attained while achieving a minimum of unit resolution at a *m/z* value of 300 atomic mass units (a.m.u.) (resolution = 300). For the present study, resolution values of between 750 and 1,000 easily fulfilled this criterion. Narrow mass scans in the continuum mode of data acquisition were performed over a mass range spanning 320 to 270 a.m.u. at a scan rate of 0.4 s with a interscan delay of 0.1 s. Alternatively, selected ion monitoring of mass 299 could be utilized, which provided increased sensitivity. The continuum mode of data acquisition was utilized as this is the only means to obtain "true" signal averaging.

Mass spectral optimization studies found no significant gain in sensitivity upon the addition of organic bases. This is advantageous, as reverse-phase columns are generally not stable at pH values greater than 9. Addition of water to methanol resulted in a slight loss in sensitivity with model synthetic compounds. The initial mobile phase of methanol/water/aqueous ammonia was utilized so as to resolve free fatty acids from PE. As it will be explained later, the establishment that the novel biomarker was indeed present as a free fatty acid and that no PE species were present in the glycolipid fraction lends itself to a variation of the chromatographic conditions. Conversion to a mobile phase consisting of 100% methanol would be more efficient in that the analysis time would be reduced with a slight gain in sensitivity. Tuning in the negative mode of ionization may vary. But for the present study, the voltage applied across the capillary was –2.13 kV, the high voltage lens was set at 0.2 kV, and the initial cone voltage was set at –22 V so as to promote "soft" ionization. The nitrogen drying gas and nebulizing gas flow rates were set at 300 and 25 L/h. respectively. The temperature of the ESI source was set at 120°C. The photomultiplier detector was set to its maximum gain value.

Extracted ion chromatograms at a *m/z* value of 299 were monitored. Ion currents for 299 in the 11–14 min range were diagnostic of 10-OH 18:0 in the free fatty acid form. Ion currents in the range of interest were averaged with background subtraction resulting in a diagnostic mass spectrum.

RESULTS

Evidence that the 10-OH 18:0 is a free fatty acid. As previously stated, earlier investigators found the 10-OH 18:0 localized at the *sn*-2 position of PE (5). However, utilization of the derivative ion scanning of specific parent ion capabilities of the triple quadrupole mass spectrometer in the negative ionization mode did not support this. Examination of those parent ions that result in a fragment ion corresponding to 10-OH 18:0 revealed no parents which could correspond to PE or any possible phospholipid. Secondly, neutral loss mass scans for a neutral loss of 141 of the acetone fraction in the positive mode of ionization revealed that this fraction contained no detectable quantities of PE species. PE was found to be located in the methanol (polar lipid) fraction through neutral loss mass scans. The retention behavior of 10-OH 18:0 on the HPLC/ESI/MS system was described in the experimental section. The acid 10-OH 18:0 was found to coelute in the free fatty acid region (12–14 min) of the chro-

matogram with a synthetic standard of 3-OH 20:0. Elution of the free hydroxylated fatty acids as a class was clearly resolved from PE which eluted as a class at 20–22 minutes. Additional evidence as to the true nature of 10-OH 18:0 was supplied through examination of the ion current for a *m/z* value of 299 at both high and low orifice voltages. At high orifice voltages, the ion current for 10-OH 18:0 was found to be over two orders of magnitude less than at low orifice voltages (1.63×10^8 peak height units at −22 V vs. 8.53×10^5 peak height units for −200 V). With synthetic phospholipids, the −200 V cone voltage was found to maximize the dissociation of the acyl groups of phospholipids resulting in a maximal ion current for the fatty acyl constituents at maximal orifice voltage settings. Lower cone voltages (−22 V) resulted in maximal ion currents for the intact molecular species. This observation provided additional evidence that 10-OH 18:0 existed as an intact molecular species and was not acylated to any phospholipid. The combined evidence of parent ion MS, neutral loss mass scans, HPLC retention behavior, and effects of variation of the orifice voltage clearly established 10-OH 18:0 existed not as an acyl constituent of PE but as a free fatty acid.

Evidence that the free fatty acid is an eighteen-carbon fatty acid with a hydroxyl functionality localized at the 10 position. Theoretically, several fatty acids could produce a *m/z* value of 299 in the negative ion mode of operation. Obviously, any monohydroxylated 18-carbon fatty acid with the hydroxyl group at positions 2–17 would yield a deprotonated molecular ion at 299. Other possibilities include but are not limited to a 17-carbon monoenoic fatty acid with a sulfhydryl (-SH) substituent as well as the polyenoic fatty acid 20:6n-3. Unambiguous assignment of the free fatty acid as 10-OH 18:0 was accomplished through comparison of the parent/derivative ion mass spectrum of 10-OH 18:0 with synthetically available standards (6-OH 18:0 and 12-OH 18:0). The first quadrupole acted as the mass filter, allowing only ions with a *m/z* value of 299 to pass into the second mass analyzer. Collision-induced dissociation (CID) of the parent molecule was accomplished in the hexapole collision chamber between the first and third quadrupoles in the presence of 0.5 Torr of argon at a collision energy of 30 eV. Diagnostic fragments were monitored by scanning the third quadrupole mass analyzer. Hydroxylated fatty acids produced low-energy CID fragment ions which were indicative of hydroxy-substituted fatty acids as a class at a *m/z* value of 281 (loss of H_2O from the hydroxyl moiety) and at a *m/z* value of 253 (loss of formic acid). Nonhydroxylated free fatty acids (palmitic acid) do not undergo any significant fragmentation under similar conditions. The site of the hydroxy functionality was confirmed to be in the 10 position through the examination of two charge-remote fragment ions that overlapped at a *m/z* value of 141 for 10-OH 18:0. The two charge-remote fragmentation processes were confirmed through examination of the daughter ion spectra of two other mid-chain, branched hydroxylated fatty acids, 6-OH and 12-OH 18:0, under identical conditions. Analogous peaks expected and observed for 12-OH 18:0 were at *m/z* values of 113 and 169. For 6-OH 18:0, fragment ions at *m/z* values of 197 and 85 were observed. The nature of the two charge-remote fragmentation events has been discussed (10). However, to summarize, fragmentation of hydroxylated fatty acids under low-energy conditions leads to fragmentation events that are distinct from previously published high-energy CID processes (11). Also, those low-energy CID fragments which are produced are indicative of hydroxylated fatty acids as a class as well as of the site of hydroxysubstitution.

Sources of 10-OH 18:0. The identification of a fatty acid unique to species of *Cryptosporidium* by Schrum *et al.* (5) was critical to the application of this technique toward complex environmental matrices. However, conventional GC/MS methodologies lack the sensitivity necessary for use of this technique as a method capable of detecting an infectious dose of *Cryptosporidium.* It was hoped that through the application of the more sensitive technique of ESI MS, a more sensitive assay might result. Also, through the identification of those endogenous 10-OH 18:0 acylated phospholipids, a great deal of specificity to the assay should be gained. Identification of the 10-OH 18:0 as a free fatty acid as opposed to an acylated component of PE was initially thought to be a fortuitous event as hydroxylated free fatty acids are extremely rare in nature. The free fatty acid nature of the biomarker was subsequently exploited for expeditious recovery and analysis by HPLC/ESI/MS with the expected gains in method sensitivity and specificity.

It was only after many repetitions of this assay that the true origin of 10-OH 18:0 was discovered. Quantities of 10-OH 18:0 extracted from oocysts of *Cryptosporidium* fluctuated wildly. Occasionally, no 10-OH 18:0 was detected from extracted oocysts of *Cryptosporidium*. The random appearance of 10-OH 18:0 in one of our sample blanks was the first indication that this compound was generated through sample handling. The observation of free 10-OH 18:0 in a sample blank was initially thought to have arisen from some interfering contaminant. However, formation of the trimethylsilyl ether adduct of the methyl ester of the sample blank resulted in an analyte possessing chromatographic behavior and a mass spectrum identical to that analyte previously described by Schrum *et al.* (5) as 10-OH 18:0.

An additional benefit of adapting the identification of the target analyte to HPLC/ESI/MS was the decrease in sample handling. This should result in a much more rapid assay with less risk of sample contamination due to the decrease in sample handling. The extraction procedures are simple and straightforward. The method involves the liquid/liquid extraction of organic soluble lipids followed by the class fractionation of neutral, glyco-, and polar lipids over silicic acid columns. The simplicity of the assay eased the efforts to localize the source of contamination once 10-OH 18:0 was realized to be an artifact of sample isolation and preparation.

All organic solvents (methanol, chloroform, acetone) used in the assay were concentrated from an initial volume of 100 mL to a final volume of 100 μL and injected onto the HPLC/ESI/MS system. Only HPLC-grade solvents were used for the extraction and fractionation of samples. All organic solvents were found to contain no 10-OH 18:0. The 10 mL of

phosphate buffer used in the liquid/liquid extraction procedures was extracted by the method of Bligh and Dyer (8), as was 10 mL of Millipore (Milford, MA) water. They, too, were found to contain no 10-OH 18:0. This eliminated the source of contamination as arising from the solvents utilized for extraction and fractionation.

Those soaps and sterilization agents utilized to clean glassware were also examined; 1 mL of Fisherbrand solvent-free concentrator, 1 mL of Fisherbrand vesphenne II, and 1 mL of a 1:1 (vol/vol) mixture of the two were extracted by the method of Bligh and Dyer (8). No 10-OH 18:0 was identified from any of these three samples.

The disposable amber rubber pipette bulbs (Fisherbrand 14 065B) were then examined. A set of five rubber bulbs was extracted, and all were found to contain authentic free acid 10-OH 18:0. This was confirmed through both HPLC/ESI/MS analysis and GC/MS separation and identification of the trimethylsilyl ether adducts of the methyl ester of 10-OH 18:0.

So as to eliminate the possibility that 10-OH 18:0 was also a lipid component of *Cryptosporidium* species as well as the rubber bulb, two samples of 1×10^8 oocysts of *Cryptosporidium* previously found to contain 10-OH 18:0 were extracted. At no time were disposable rubber bulbs utilized. Both sets of experiments were found to contain no detectable quantities of 10-OH 18:0 through HPLC/ESI/MS and GC/MS analysis when disposable rubber bulbs were not used in the manipulations. This established that the target analyte was not an interfering contaminant but an artifact of the sample preparation procedures.

DISCUSSION

The putative occurrence of such an unusual component as 10-OH 18:0 in *Cryptosporidium* (4,5) led us to investigate the complex native form of this fatty acid in *C. parvum*. When the new studies by R.S. Burkhalter and C.A. Smith showed that the 10-OH 18:0 was a free fatty acid and not as an ester in PE (6), it was then realized that the fast atom bombardment (FAB) mass spectrum of PE presented by Schrum *et al.* (5) actually provided no evidence supporting the presence of 10-OH 18:0 as an acyl constituent in PE. This spectrum was obtained under positive ionization conditions, which will not produce fragment ions corresponding to fatty acyl constituents. Positive-ion FAB MS should provide protonated molecular ions and diglyceride ions after loss of the polar head group as a neutral species. FAB MS performed in the negative ion mode of operation would be expected to generate fatty acids with charge retention after acyl cleavage. In short, the fragment ion at 299 in the positive ion FAB mass spectrum of the major PE did not correspond to a fatty acyl constituent 10-OH 18:0. The enzymatic hydrolysis of PE recovered by thin-layer chromatography and subsequent derivatization with electron-withdrawing groups for negative ion detection by GC/MS (6) achieved the sensitivity to detect 10-OH 18:0, but the 10-OH 18:0 was apparently added during the preparation as a contaminant and was not in the PE.

When adaptation of HPLC/ESI/MS techniques revealed 10-OH 18:0 to be a free fatty acid, the putative signature became even more unique. Efforts were focused to characterize the target analyte of interest more completely and to establish lower limits of detection. Difficulty in producing a calibration curve for oocyst number vs. content of 10-OH 18:0 became a major problem. This was rationalized as being due to sample heterogeneity after microscopic examination of the preparations showed the oocysts clumped together and attached to fibers that were in these purified fecal preparations. The clumping increased with storage at 4°C. Difficulty in dispersing the oocysts reproducibly was thought to be the problem. Unfortunately, the random nature of the appearance of the 10-OH 18:0 prevented the appearance of the analyte in a sample blank until many repetitions were performed. Clearly when examining for traces of specific analytes such as found in 10–30 oocysts, comprehensive blank analyses which test for each step of the procedure must be rigorously performed. If repeated controls of lipids from a bacterium known not to contain 10-OH 18:0 had been utilized, the artifact might have been detected earlier.

Lipid biomarkers and Cryptosporidium *oocyst infectivity.* There is reproducible evidence of a correlation between loss of infectivity and oocyst metabolism in *C. parvum*. R. Fayer (personal communication; USDA, ARS, LPSI Parasite Immunology Laboratory, Beltsville, MD) has shown a direct correlation between the loss in amylopectin content of *C. parvum* oocysts and infectivity to BALB/c neonatal mice. There is a greater than threefold decrease in polar lipid fatty acids (PLFA) (from 3.8 to 0.75 pmoles/10^3oocysts) with a shift in patterns (4). With loss of infectivity by freezing there is a striking decrease in the proportion of 18:1n-9*c*, an increase in 18:2n-6 and 20:3n-6 with little change in the proportion of 16:0. During freezing of *C. parvum* oocysts, the phospholipids and cholesterol decrease, polyenoic PLFA accumulate, and polyenoic fatty acids in the polar lipids and neutral lipids increase. Lipid shifts have been shown to be excellent markers for the nutritional/physiological status in bacteria (12). Identification of lipid shifts with infectivity is a tool that will be useful in research studies of infectivity, but overlap in lipid composition with algae and other components of drinking water community requires that the oocytes be highly purified before analysis prior to use as a monitoring system for infectious *Cryptosporidium*. Patterns of PLFA of purified oocysts can used to differentiate between *C. parvum* and the nonhuman pathogens *C. baileyi, C. serpentis*, and *C. muris* (6).

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