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PAPER

CRIMINALISTICS

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Trace Detection of Meglumine and Diatrizoate from Bacillus Spore Samples Using Liquid Chromatography/Mass Spectrometry*,§

ABSTRACT: Following the September 11, 2001 terrorist attacks, letters containing *Bacillus anthracis* were distributed through the United States postal system killing five people. A complex forensic investigation commenced to identify the perpetrator of these mailings. A novel liquid chromatography/mass spectrometry protocol for the qualitative detection of trace levels of meglumine and diatrizoate in dried spore preparations of *B. anthracis* was developed. Meglumine and diatrizoate are components of radiographic imaging products that have been used to purify bacterial spores. Two separate chromatographic assays using multiple mass spectrometric analyses were developed for the detection of meglumine and diatrizoate. The assays achieved limits of detection for meglumine and diatrizoate of 1.00 and 10.0 ng/mL, respectively. *Bacillus cereus* T strain spores were effectively used as a surrogate for *B. anthracis* spores during method development and validation. This protocol was successfully applied to limited evidentiary *B. anthracis* spore material, providing probative information to the investigators.

KEYWORDS: forensic science, meglumine, diatrizoate, *Bacillus anthracis*, *Bacillus cereus*, liquid chromatography/mass spectrometry

In the weeks following the September 11, 2001 terrorist attacks on the World Trade Center and the Pentagon, four letters containing *Bacillus anthracis* spores were collected. These letters were addressed to two media outlets in New York City and to two members of the United States Senate in Washington, DC. As a result of the distribution of these letters through the United States Postal System, five victims died and at least 17 victims demonstrated symptoms of inhalational or cutaneous anthrax (1).

The investigation to determine the individual(s) responsible for the most disruptive terrorist attack on the United States involving the use of a biological agent was conducted by the Federal Bureau of Investigation (FBI) and the United States Postal Inspection Service (USPIS). Because of unprecedented challenges, this was among the most complex investigations the FBI or the USPIS had ever conducted. The investigation team, known as the Amerithrax Task Force, worked with subject matter experts and the scientific community to develop novel analytical/forensic assays to leverage all possible information from the evidentiary *B. anthracis* spore materials.

The *B. anthracis* spores recovered from the letters were determined to be of the Ames strain and were of a high degree of purity. While the spores recovered from the mailings to media outlets in New York City were characteristically different (e.g., off-white in color, more granular, some cellular debris, and growth media components present) from the spores sent to Washington, DC., both had high colony-forming units (CFU) per gram of material, on the order of 10^{11} CFU/g, indicating that both were high-quality spore preparations. Spores of such purity are often used in conducting aerosol challenges (2) to minimize the incidence of nebulizer obstruction by cellular debris or growth media components during an experiment.

The investigation determined that some laboratories conducting *B. anthracis* research with the Ames strain were purifying spores using a density gradient of RenoCal-76® or similar products. Meglumine diatrizoate and sodium diatrizoate are the primary constituents in RenoCal-76® (Fig. 1), Hypaque-76®, and Renografin-60®, which are commercially available radiographic imaging products. In addition, meglumine diatrizoate, meglumine, and sodium diatrizoate are readily available from commercial chemical suppliers. The literature reports, as early as 1966, the use of products containing meglumine diatrizoate in spore purification (3,4).

The Ames *B. anthracis* used in the New York City and Washington, DC mailings had a number of identified morphological variants, which were isolated and their complete genomes sequenced. The sequences of these variants were compared to the wild-type Ames *B. anthracis*, and a number of genetic differences were identified (5). Assays were used to screen over 1000 samples of Ames *B. anthracis* collected from research institutions within the United

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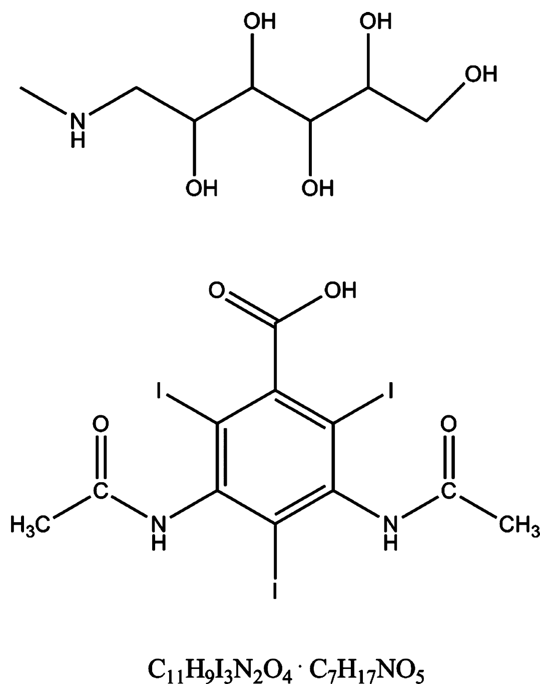

FIG. 1—*Meglumine diatrizoate.*

States and internationally. Of the samples screened, all samples positive for all of the genetic markers were determined to originate from a common source of spores, known as RMR-1029. The RMR-1029 spores were known, from laboratory records, to have been purified using a density gradient of RenoCal-76®. Some investigative questions became: "Were the evidentiary spores from the mailings directly diverted from RMR-1029? Could an analytical method identify residual RenoCal-76® in a spore preparation known to be purified using RenoCal-76®?"

This paper describes the development, validation, and application of a novel, highly sensitive protocol using liquid chromatography/mass spectrometry (LC/MS) with electrospray ionization (ESI) to detect trace amounts of meglumine and/or diatrizoate, components of RenoCal-76®, in a single spore sample preparation. This analytical capability was applied to limited evidentiary spore material and RMR-1029 to provide probative information about a possible production method of the evidentiary samples. During the investigation, it was determined that the number of researchers who used gradients of RenoCal-76®, or similar products, to purify spores and who had access to the Ames strain of *B. anthracis* was limited. Therefore, if meglumine and diatrizoate were identified in the *B. anthracis* spores used in the mailings, the number of potential sources for the spore material could be significantly reduced.

Materials and Methods

Chemicals

Mass spectrometry (MS) grade solvents were commercially available and used without further purification. Reagent grade ammonium hydroxide was purchased from Thermo Fisher Scientific (San Jose, CA). Reagent grade formic acid (88%) was purchased from J.T. Baker (Phillipsburg, NJ). High-purity water was purchased from Burdick & Jackson (Morristown, NJ). RenoCal-76® was obtained from Bracco Diagnostics Inc. (Princeton, NJ). Meglumine diatrizoate, D-sorbitol, and phenol were purchased from Sigma Aldrich (St. Louis, MO). Omnipaque® (Iohexol) was obtained from Amersham Health (Princeton, NJ), and Metrizamide® was purchased from Fitzgerald Industries International Inc. (Acton, MA). Leighton–Doi medium (6) and phosphate-buffered saline were freshly prepared using commercially available chemicals. Blood agar plates (trypticase soy agar with 5% sheep blood) were purchased from Remel (Lenexa, KS).

High-Performance Liquid Chromatography Conditions

All chromatographic separations were isocratic and performed using an Agilent 1100 series (Santa Clara, CA) high-performance liquid chromatography (HPLC) system comprised of a solvent degassing unit, binary pump, and autosampler. An Alltech Alltima C18 (2.1 × 150 mm; 5 μm, part number 88370; Nicholasville, KY) column was used for diatrizoate analyses with a flow rate of 0.2 mL/min and a run time of 5 min. The mobile phase consisted of 95:5 methanol/water and 0.03% ammonium hydroxide with an approximate pH of 8. The autosampler needle was washed with methanol after each injection. A Thermo Fisher Scientific Hypercarb (2.1 × 100 mm, 5 μm, part number 35005–102130) column was used for the meglumine analyses with a flow rate of 0.2 mL/min and a run time of 5 min. The mobile phase consisted of 80:20 acetonitrile/water and 0.02% formic acid with an approximate pH of 4. The autosampler needle was washed with acetonitrile after each injection. All analyses were conducted at ambient temperature.

Mass Spectrometer Conditions

MS analysis was conducted using an LTQ mass spectrometer (Thermo Fisher Scientific), equipped with an ESI source and a Rheodyne injector. All data were analyzed using Xcalibur software (San Jose, CA). As a result of having separate LC conditions for meglumine and diatrizoate, the mass spectrometer settings were optimized for each component. Specific tune files were developed utilizing the autotune function in Xcalibur while infusing a 5.00 μg/mL solution of meglumine diatrizoate (combined with the appropriate LC mobile phase) with a set mass of 196.1 *m/z* for meglumine and a set mass of 631.7 *m/z* for diatrizoate, which reflects an ammonium adduct. The following mass spectrometer conditions were applied: positive ion mode; capillary temperature, 275°C; sheath gas, 20 units; auxiliary gas, 5 units; and spray voltage, 4.50 kV. Analyte specific fine tuning of the capillary and split lens voltages was performed and tune files were saved and applied during the appropriate analyses.

Direct Injection Full-Scan MS

In these experiments, sample was manually injected into a 5-μL sample loop of the Rheodyne injector. The valve was manually rotated to introduce the 5-μL of sample into the established LC conditions. ESI was performed in positive ion mode, and full-scan spectra were acquired across a scan range of 100–850 *m/z*. The syringe and sample loop were manually rinsed several times with high-purity water in between injections, and blank injections of high-purity water were conducted during the analyses to demonstrate the absence of carryover.

Full-Scan LC/MS

In these experiments, a 5-μL sample was injected via an autosampler and eluted using the established chromatographic

conditions. ESI was performed in positive ion mode, and full-scan spectra were acquired across a scan range of 100–850 *m/z*.

LC/MS^2

In these selected reaction monitoring (SRM) experiments, a 5-μL sample was injected via an autosampler and eluted using the established chromatographic conditions. ESI was performed in positive ion mode, and the MS was operated in SRM mode. For meglumine, the selection mass range was 195.4–196.9 *m/z* and the monitoring mass range was 177.7–178.7 *m/z*. To capture the associated adduct ions of diatrizoate, the selection mass range was 612.0–638.0 *m/z*, and the monitoring mass range, 360.5–361.5 *m/z*. The normalized collision energy was 35%, activation Q (radio frequency) was 0.250, activation time (fragmentation time) was 31.0 msec, and the isolation width was 26.0 *m/z* for diatrizoate and 1.5 *m/z* for meglumine.

LC/MS^4

In these experiments, 5 μL of sample was injected via an autosampler and eluted using the established chromatographic conditions. ESI was performed in positive ion mode, and the MS was operated in continuous reaction monitoring mode. For meglumine, the primary selection mass range was 195.4–196.9 *m/z*, the secondary selection mass range was 177.4–178.9 *m/z*, the tertiary selection mass range was 159.4–160.9 *m/z*, and the final monitoring mass range was 73.5–74.5 *m/z*. For diatrizoate, the primary selection mass range was 612.0–638.0 *m/z*, the secondary selection mass range was 360.5–361.5 *m/z*, the tertiary selection mass range was 232.6–233.6 *m/z*, and the final monitoring mass range was 147.6–148.6 *m/z*. During the MS^4 transition, the normalized collision energy was 35%, Q was 0.250, time was 30.0 msec, and the isolation width was 1.0 *m/z* for diatrizoate and 1.5 *m/z* for meglumine.

Spore Preparation

B. anthracis is a virulent pathogen that is commonly handled under biosafety level 3 containment. To minimize the risk of personnel developing the assay, *Bacillus cereus* T strain spores were used as a surrogate for *B. anthracis* during the development and validation of the assay. This strain of *B. cereus* is morphologically similar to *B. anthracis*, having highly similar spore structural characteristics such as an exosporium (7). *B. cereus* can be safely handled under biosafety level 2 containment.

The protocol for spore production and purification has been previously reported (2,8). Briefly, *B. cereus* spores were inoculated on trypticase soy agar with 5% sheep blood and incubated overnight at 37°C. An inoculating loop was used to pick 2–5 colonies that were resuspended into 10 mL of phosphate-buffered saline, of which 50–100 μL was used to inoculate 250 mL of Leighton–Doi media contained in a 1-L flask. The cultures were incubated at 37°C for 24 h, shaking at 300 strokes per minute (SPM), then at room temperature for 3 days while shaking at 300 SPM. Sporulation was determined to be 98% using phase-contrast microscopy. Spores were harvested by centrifugation at 7000 × *g* for 20 min at 4°C. The resulting supernatant was removed, and the pellet was resuspended in 25 mL of sterile water. The centrifugation step was repeated. After the supernatant was removed, the spore pellet was washed once again with sterile water. The supernatant was removed, and the final pellet was resuspended in a sterile 1% phenol solution and stored at 4°C.

Spore Purification

The spore suspension (26 mL) was layered on top of a 58% solution of RenoCal-76® (100 mL) in a 500-mL centrifuge bottle and centrifuged at 8000 × *g* for 2 h. The solution was decanted from the bottle to leave the spore pellet, which was resuspended and run through a second 58% solution of RenoCal-76®. The final spore pellet was washed by resuspending in 1% phenol and centrifuged at 8000 × *g* for at least 15 min. The supernatant was removed by pipetting, and the wash step was repeated a second time. Once the final supernatant was removed, the resulting pellet was resuspended in sterile 1% phenol and stored at 4°C.

A portion of the *B. cereus* spores was dried overnight using a SpeedVac. In an effort to mimic the processing of the evidentiary samples to be analyzed by this protocol, an aliquot of the dried *B. cereus* spores, on wet ice, was exposed to 4×10^6 Rads of gamma radiation with a cobolt 60 source. Irradiation has been described as an effective means of inactivation of the virulent pathogenic activity of the *B. anthracis* (9).

Spore Extraction

Approximately 2 mg of spore material was transferred into a 1.5-mL Eppendorf tube and 200 μL of 18 MΩ (or equivalent high-purity) water was added. Each sample was vortexed for 5 min at room temperature and then submerged into a sonicating water bath for 5 min at room temperature. Each sample was briefly vortexed again to ensure uniform dispersion. Samples were centrifuged at 16,000 × *g* at room temperature for 10 min, and the supernatant was transferred into a vial and analyzed using the LC/MS parameters described above.

Evidentiary samples from the investigation as well as blinded control samples that included the RMR-1029 sample were submitted to the FBI Laboratory for analysis. The control samples were submitted as single-blinded samples to the FBI scientists and were hence treated as unknown samples.

Results

RenoCal-76® is a solution that contains 66% meglumine diatrizoate and 10% sodium diatrizoate. At concentrations <50 μg/mL meglumine diatrizoate, the meglumine diatrizoate salt dissociates to meglumine 196 *m/z* $[M+H]^+$ and diatrizoate 614 *m/z* $[M+H]^+$, 632 *m/z* $[M+NH_4]^+$, and 637 *m/z* $[M+Na]^+$. The 196 *m/z* $[M+H]^+$ was associated with the molecular ion of meglumine. The diatrizoate ions of 632 *m/z* $[M+NH_4]^+$ and 637 *m/z* $[M+Na]^+$ were consistent with ammonium and sodium adducts of the diatrizoate, respectively. The molecular ion of diatrizoate 614 *m/z* $[M+H]^+$ was less abundant. The experimental methods described in this article were developed, evaluated, and validated with an increased isolation width of 26.0 *m/z* to encompass all of the diatrizoate-related ions.

During method development, it was recognized that both meglumine and diatrizoate chromatographically elute and ionize using alkaline LC condition; however, there was persistent carryover with meglumine under the alkaline conditions. After investigating additional autosampler needle washes and incorporation of blank injections, it was ultimately decided to develop separate LC conditions for the analysis of meglumine, thereby significantly minimizing the carryover issues. Additionally, the use of separate LC conditions enabled the optimization of specific MS parameters for each component to maximize instrument sensitivity.

In pursuit of a sensitive and selective method for unknown concentration levels of limited evidentiary material, a multiple-tiered analytical approach was developed. This innovative analytical approach uses a single preparation of only 2 mg of dried spore material. The method design includes both screening and confirmatory techniques, gathers chromatographic and MS data, and is suitable for samples that are concentrated or those at trace levels. The method validation was performed in accordance with the recommendations described by the Scientific Working Group on Forensic Analysis of Chemical Terrorism for a qualitative method (10). Limits of detection (LODs) and selectivity for meglumine and diatrizoate were determined. To conserve the limited evidentiary material, the protocol was developed and validated using a single sample preparation procedure, followed by multiple injections using the tiered LC/MS methods, described below, for both meglumine and diatrizoate.

The LOD was determined by analyzing increasing concentrations of aqueous meglumine diatrizoate standards over a range 1.00 pg/mL to 50.0 μg/mL. The large range was selected to empirically capture an LOD across all four MS analyses. The LOD was defined as a signal that possessed a peak height that was >3 times that of the noise. The noise was calculated from comparable retention time windows of the water blanks that immediately preceded the standard. The LOD analyses included triplicate injections at each concentration, using the multiple-tiered analytical approach. Analyses were repeated across three separate analytical run days. All data were reviewed according to the decision criteria described *infra* to determine the LOD for each experiment and each analyte.

The first-tier experiments consisted of a direct injection, full-scan MS method. A meglumine molecular ion of 196 *m/z* $[M+H]^+$ was observed using the established meglumine conditions (Fig. 2). The diatrizoate ions of 632 *m/z* $[M+NH_4]^+$ and 637 *m/z* $[M+Na]^+$ were detected using the established diatrizoate conditions (Fig. 3). Results from the direct injection full-scan MS would immediately indicate whether levels greater than the LOD of 1.00 μg/mL for meglumine and/or diatrizoate were present in the unknown sample. Comparison of the peak response of an unknown sample to the response of the positive controls would indicate which samples may need dilution. Unknown samples with a response greater than two times the response of the positive controls would be considered for dilution with purified water prior to introducing the sample into the LC system in an effort to avoid unwanted carryover.

The second-tier experiments incorporated a chromatographic separation prior to a full-scan MS analysis. LODs were determined to be 0.100 and 1.00 μg/mL for meglumine and diatrizoate, respectively. For meglumine, a molecular ion of 196 *m/z* $[M+H]^+$ was observed at a retention time of 0.99 min (Fig. 4). Ammonium and sodium adduct ions of diatrizoate with corresponding masses of 632 *m/z* $[M+NH_4]^+$ and 637 *m/z* $[M+Na]^+$, respectively, were observed at a retention time of 1.06 min (Fig. 5). The addition of the orthogonal approach of LC provided increased selectivity for the target analytes. Capturing full-scan mass spectral data using both acidic and alkaline LC conditions enabled detection of the compounds of interest and also the detection of additional unknown constituents that may elute and ionize under these conditions. It was understood that unknown samples containing low levels of meglumine (≤0.100 μg/mL) and diatrizoate (≤1.00 μg/mL) may not give positive results (i.e., observation of expected ions) for the compound(s) of interest during the first- and second-tier experiments. Thus, a negative result from these tiers would not impact the decision criteria for the procedure.

The third- and fourth-tier experiments focused on enhancing sensitivity and selectively and would impact the decision criteria of the assay. The third-tier experiments were LC/MS^2 methods and resulted in LODs of 1.00 and 10.0 ng/mL for meglumine and diatrizoate, respectively. The transition of 196 → 178 *m/z* was monitored for meglumine, and the transition of 612–638 → 361 *m/z* was monitored for diatrizoate (Fig. 6(*a*) meglumine (*b*) diatrizoate). Finally, the fourth-tier experiments were LC/MS^4 methods that provided increased selectivity. (Note: the MS^2 and MS^3 fragmentations resulted in only losses of water.) The following transition was monitored for meglumine (LOD of 100 ng/mL); 196 → 178 → 160 → 74 *m/z* (Fig. 7*a*). The following transition was monitored for diatrizoate (LOD of 10.0 ng/mL); 612–638 → 361 → 233 →

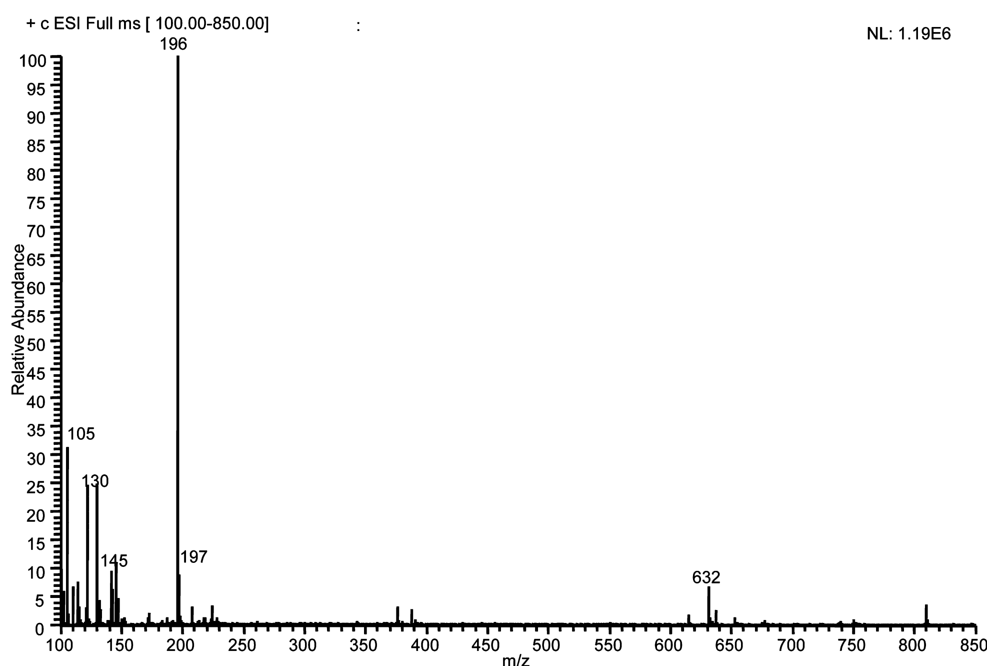

FIG. 2—*Full-scan MS analysis (100–850 m/z) of meglumine diatrizoate (5 μg/mL) using instrument conditions optimized for meglumine (196 m/z).*

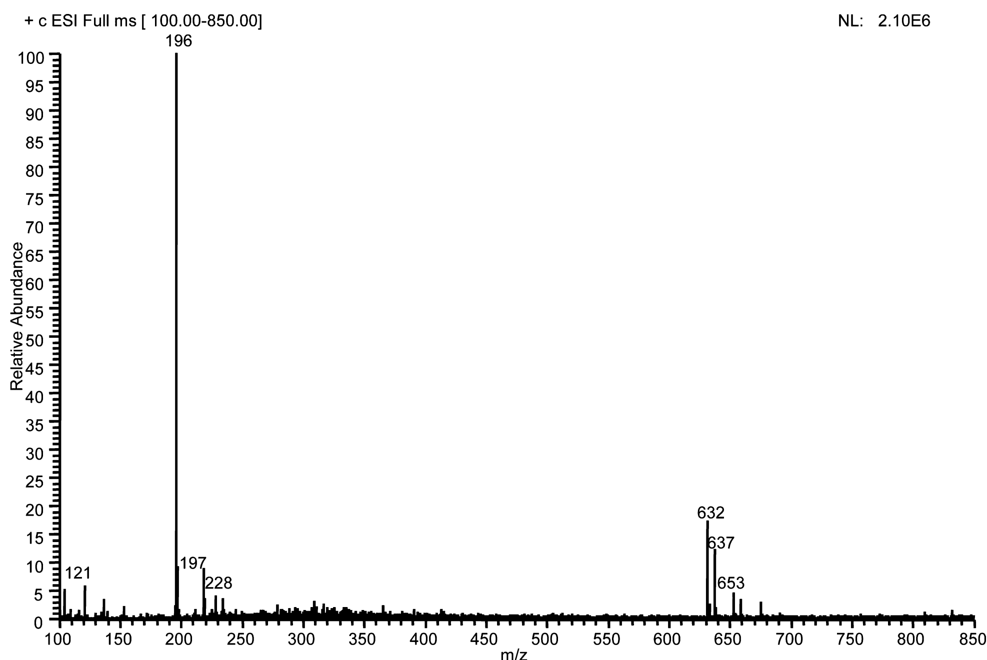

FIG. 3—*Full-scan MS analysis (100–850) of meglumine diatrizoate (5 μg/mL) using instrument conditions optimized for diatrizoate (632 m/z, 637 m/z).*

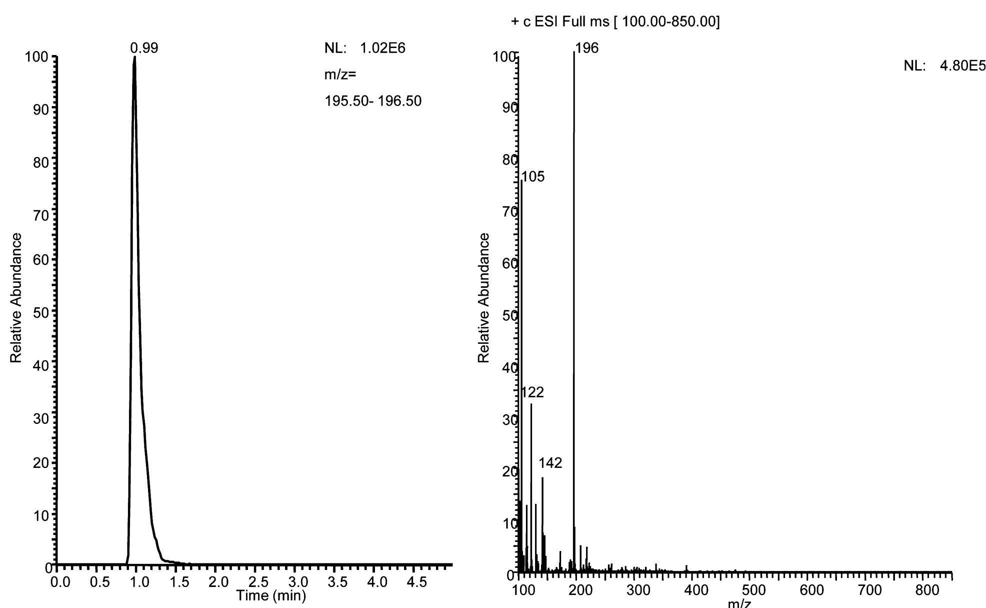

FIG. 4—*Extracted ion chromatogram for meglumine (196 m/z). Full-scan liquid chromatography/mass spectrometry analysis (100–850 m/z) of meglumine diatrizoate (5 μg/mL) using instrument conditions optimized for meglumine.*

148 *m/z* (Fig. 7*b*). The LC/MS^2 and the LC/MS^4 results demonstrated the most sensitivity and selectivity, respectively, for the assay. Hence, the ions described above for the third- and fourth-tier experiments should be observed for an unknown sample to be considered as containing the components of meglumine/diatrizoate.

The selectivity of the method was evaluated through the analysis of positive and negative controls. Positive controls included aqueous meglumine diatrizoate solutions; aqueous extracts of RenoCal-76®-purified, gamma-irradiated *B. cereus* spores; and aqueous extracts of non-RenoCal-76®-purified, gamma-irradiated *B. cereus* spores extracted with a 5.00 μg/mL solution of meglumine diatrizoate in water. The negative controls evaluated included high-purity water and aqueous extracts of non-RenoCal-76®-purified, gamma-irradiated *B. cereus* spores. Selectivity was further demonstrated by the analysis of a number of chemicals similar to meglumine and diatrizoate. Reference solutions of sorbitol, metrizamide, and Iohexol (Omnipaque® contains 517.7 mg/mL Iohexol) at 5.00 μg/mL were prepared and analyzed. The expected ions of $[M+H]^+$, $[M+NH_4]^+$, and $[M+Na]^+$ were observed during the full-scan analyses, and no interfering ions were detected during the LC/MS^2 and LC/MS^4 analyses.

Decision criteria were established to consistently evaluate the data generated from this protocol. As previously mentioned, full-scan MS data were used to primarily evaluate sample concentration of

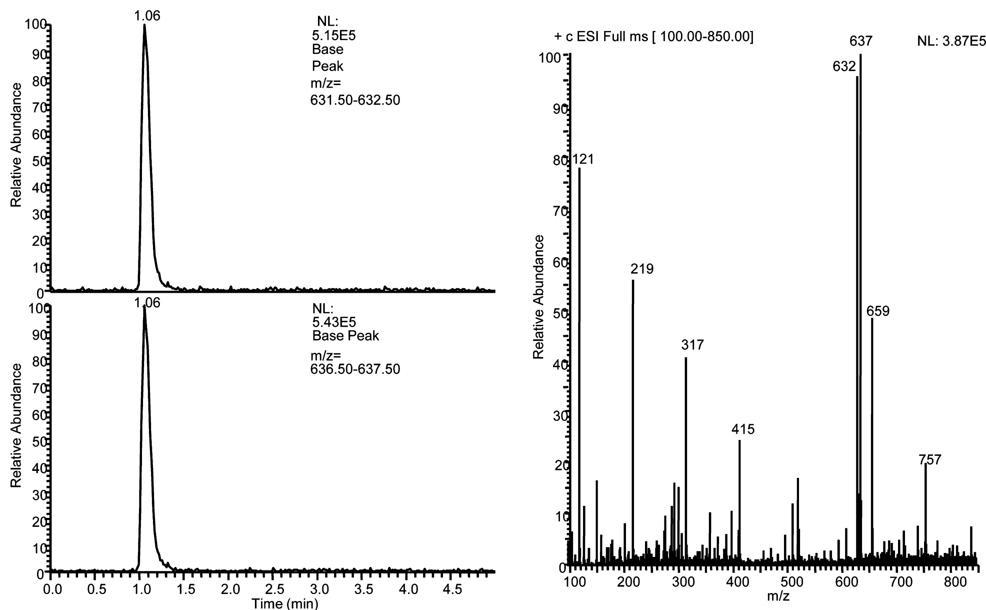

FIG. 5—*Extracted ion chromatograms for diatrizoate (632 and 637 m/z). Full-scan liquid chromatography/mass spectrometry (100–850 m/z) analysis of meglumine diatrizoate (5 μg/mL) using instrument conditions optimized for diatrizoate.*

meglumine diatrizoate to avoid overloading the LC system and to capture full-scan data. Samples that contain very dilute amounts of meglumine diatrizoate may not give positive results during the full-scan MS analyses. Thus, negative results from these two assays would not prohibit continuation with LC/MS^2 and LC/MS^4 analyses.

The LC/MS^2 and LC/MS^4 experiments provided highly sensitive and selective data for detecting the presence of the meglumine and diatrizoate. If samples gave positive results for the full-scan MS analyses, then expected ions for LC/MS^2 and LC/MS^4 should also be observed. At a minimum, a positive result for LC/MS^2 and LC/MS^4 must be satisfied to determine that a sample contained meglumine and/or diatrizoate. Additionally, chromatographic data generated should meet the following criteria: Gaussian peak shape, a signal-to-noise ratio for the peak(s) of interest of at least 3:1, and retention times of the analytes within ±5% of an appropriate positive control. These criteria were not empirically determined, but rather reflect a typical approach used by the FBI Laboratory during the evaluation of chromatographic data.

B. anthracis evidence must be gamma-irradiated to inactivate the virulent spores prior to submission to the FBI Laboratory for analysis. During the method development of this assay, RenoCal-76®-purified *B. cereus* spores were irradiated under similar conditions used to inactivate the *B. anthracis* evidence. The irradiated spores were then analyzed to confirm the presence of meglumine and diatrizoate. The results demonstrated that irradiation of the spores had no adverse effects on the ability of the assay to detect either meglumine or diatrizoate.

As previously mentioned, the investigation was complex and many forensic analyses were conducted. To further minimize consumption of evidence, investigators requested this assay be developed using a minimal amount of spore material. As a result, the method was developed and validated using *c.* 2 mg of irradiated dry spore material. It is appropriate to highlight the difficulty encountered in obtaining an accurate weight of such a small amount of dried spore material owing to the electrostatic properties. A zerostat antistatic instrument and antistatic vinyl gloves were used during the manipulations of any dried spore material. Weighing small quantities of spore material proved to be challenging and may have been problematic had a quantitative assay been pursued.

Application to Evidentiary Spore Samples

For each tier of the assay, the following negative controls were included: negative control water and an extract of negative control spores of the *B. cereus* spores prepared in the absence of RenoCal-76®. Positive control samples were also included in the analyses and consisted of extracts from *B. cereus* spores purified with RenoCal-76® and a 5.00 μg/mL reference solution of meglumine diatrizoate. Blank water injections were conducted in between control samples and in between each unknown sample injection to monitor carryover.

An analysis of RMR-1029 using the validated, multiple-tiered approach described herein resulted in a positive response in all assays for meglumine and a positive response for LC/MS^2 and LC/MS^4 for diatrizoate. These data confirmed that residual meglumine and diatrizoate were identified on the RMR-1029 spores. When the meglumine and diatrizoate assays were applied to the evidentiary spore material, neither meglumine nor diatrizoate was detected within the LODs.

Discussion

The purification of *B. anthracis* spores with products like RenoCal-76® has been previously reported to be an effective means of purifying spores (3,4); however, it is appropriate to mention that it is not considered the standardized approach. RenoCal-76®, Hypaque-76®, and other similar products have a clinical use in the radiographic imaging field and are not commercially available to research laboratories for the purposes of spore purification. The investigation determined that some laboratories conducting *B. anthracis* research, with the specific strain identified in the evidence, were using RenoCal-76® or similar products to purify spores. It was believed by the Amerithrax Task Force that trace levels of RenoCal-76®, or its primary components of meglumine diatrizoate and

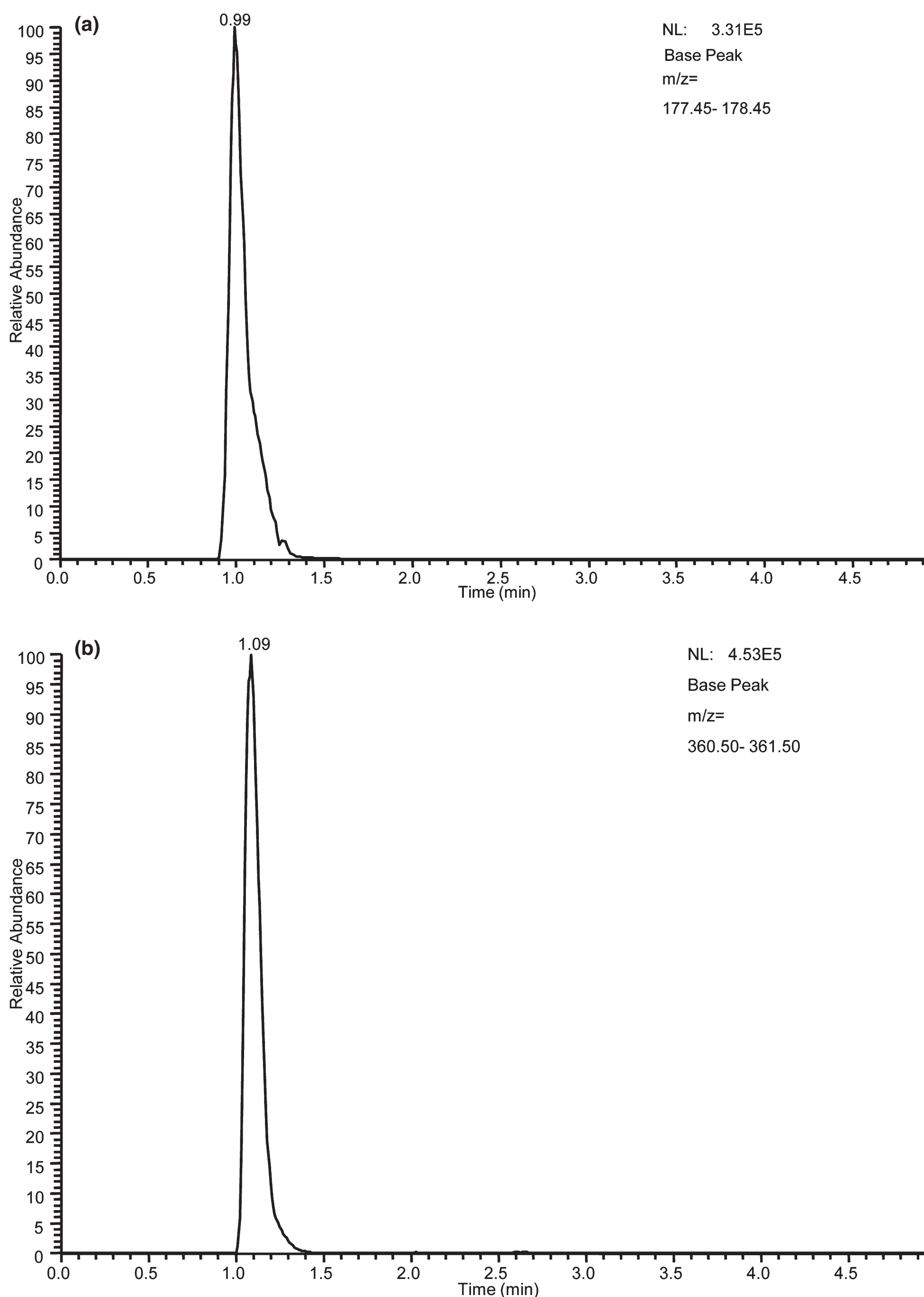

FIG. 6—*(a) LC/MS² analysis of meglumine diatrizoate (5 μg/mL). Monitoring the 196 → 178 m/z transition using instrument conditions optimized for meglumine. (b) LC/MS² analysis of meglumine diatrizoate (5 μg/mL). Monitoring the 612–638 → 361 m/z transition using instrument conditions optimized for diatrizoate.*

sodium diatrizoate, may persist on the outer spore surface. The primary objective of the work described in this manuscript was to develop and validate a sensitive and selective assay for the qualitative detection of meglumine and diatrizoate. Potential source information is always valuable in a forensic investigation. Although these results did not confirm the possible source of the spore material, they did indicate that the spore material used in the mailings was consistent with not being diverted directly from RMR-1029.

Meglumine diatrizoate is a component of several of the radiographic imaging products like RenoCal-76®, Hypaque-76®, and Renografin-60®. Although LC/MS conditions for diatrizoic acid have been reported in the literature (11,12), the novel assay described herein supplies LC/MS conditions for diatrizoate and meglumine, as well as an extraction technique to detect these chemicals from *Bacillus* spores. The multiple-tiered approach of utilizing direct injection full-scan MS, LC/MS full scan, LC/MS^2, and LC/MS^4 with a single sample preparation provided an innovative approach to analyzing limited forensic evidence. The full-scan MS analysis offered a screening for potential samples that may overload the analytical system at the same time of capturing data of additional components that may be present. The LC/MS^2 afforded the sensitivity, and the LC/MS^4 provided the necessary selectivity. This

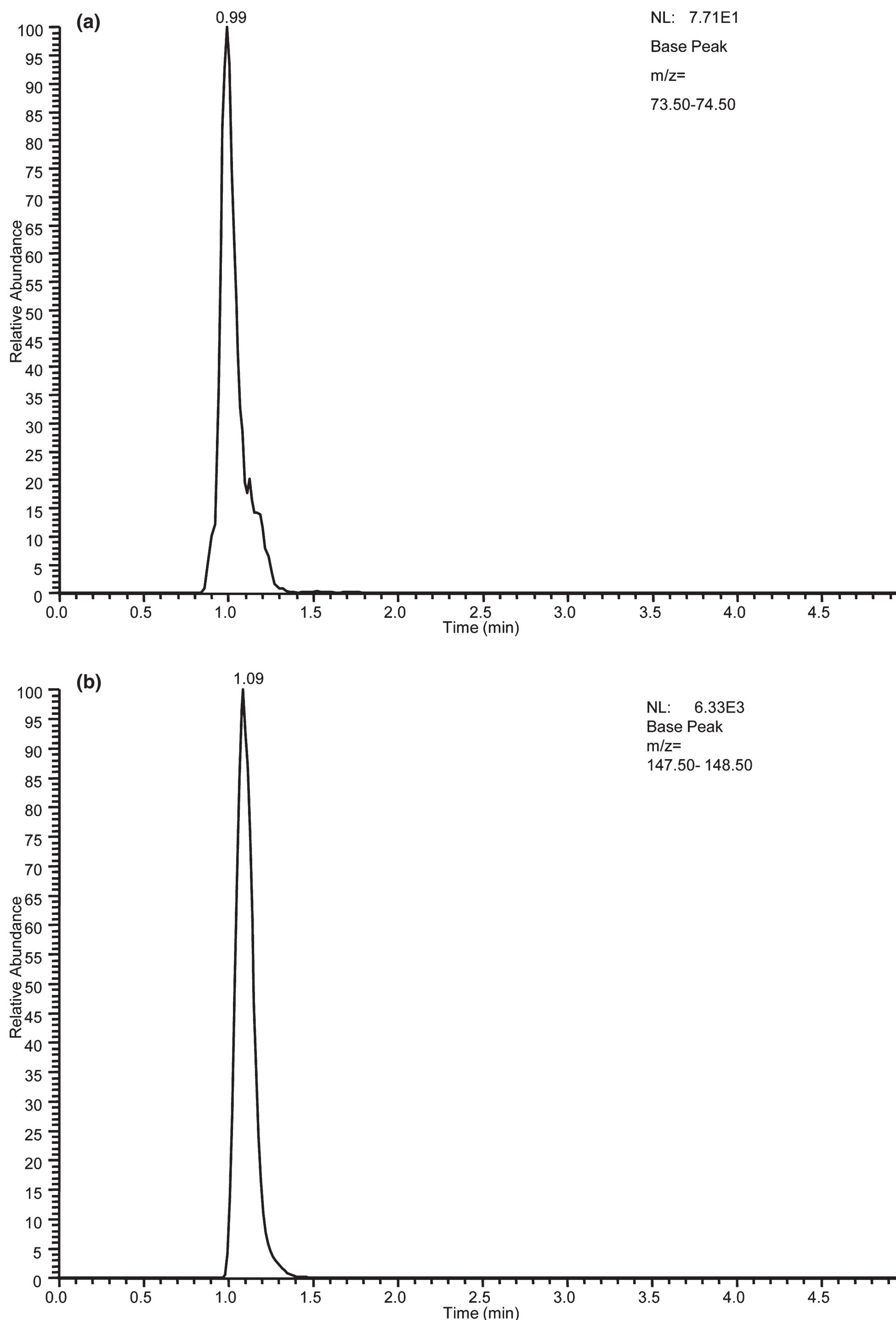

FIG. 7—*(a) LC/MS² analysis of meglumine diatrizoate (5 μg/mL). Monitoring the 196 → 178 → 160 → 74 m/z transition using instrument conditions optimized for meglumine. (b) LC/MS² analysis of meglumine diatrizoate (5 μg/mL). Monitoring the 612–638 → 361 → 233 → 148 m/z transition using instrument conditions optimized for diatrizoate.*

approach could be considered in other instances where limited evidentiary material must be thoroughly analyzed for chemicals of concern. The beneficial use of MS^3 detection on limited spore material has been previously described in the literature (13).

Future work on this method would be to explore different stationary and mobile phases, as well as gradient elution, to chromatograph both the meglumine and diatrizoate in a single analysis without carryover concerns. If both compounds could be analyzed in a single analysis, this method would be a viable option to rapidly analyze many samples in a high-throughput fashion. Additionally, efforts to develop a quantitative method for the detection of meglumine diatrizoate may be explored in the future.

Conclusion

A sensitive and selective analytical protocol has been developed for the detection of meglumine and diatrizoate in samples of *Bacillus* spores. A tiered approach of capturing chromatographic

separation, full-scan MS, MS^2, and MS^4 data was developed for both meglumine and diatrizoate. System carryover concerns with meglumine were resolved by changing the stationary and mobile phases. The method validation demonstrated both sensitivity and selectivity by obtaining detection limits of meglumine and diatrizoate at concentrations ranging from 1.00 to 10.0 ng/mL. Maximizing the data that could be derived from the analysis of a few milligrams of evidentiary material was paramount to the FBI. The application of this novel method proved to be a valuable tool during the investigation. As the genetic data that linked the *B. anthracis* spore material from the mailings to RMR-1029 was being compiled, investigators were uncertain whether an aliquot of RMR-1029 was used directly. The absence of meglumine and diatrizoate on the evidentiary material, using the protocol described herein and when taken together with other forensic examinations, was supportive to the investigation in indicating that the evidentiary spore material was not diverted directly from RMR-1029.

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References

1. Centers for Disease Control and Prevention. Update: investigation of bioterrorism-related anthrax—Connecticut 2001. Morb Mortal Wkly Rep 2001;50(48):1077–9.
2. Ivins BE, Fellows PF, Pitt MLM, Estep JE, Welkos SL, Worsham PL, et al. Efficacy of a standard human anthrax vaccine against *Bacillus anthracis* aerosol spore challenge in rhesus monkeys. Salisbury Med Bull 1996;87:125–6.
3. Tamir H, Gilvarg C. Density gradient centrifugation for the separation of sporulating forms of bacteria. J Biol Chem 1966;241(5):1085–90.
4. Wyrick PB, Brownridge EA, Ivins BE. Interaction of *Chlamydia psittaci* with mouse peritoneal macrophages. Infect Immun 1978;19(3):1061–7.
5. Rasko DA, Worsham P, Abshire TG, Stanley ST, Bannan JD, Wilson MR, et al. *Bacillus anthracis* comparative genome analysis in support of the Amerithrax investigation. Proc Natl Acad Sci USA 2011;108(12): 5027–32.
6. Leighton TJ, Doi RH. The stability of messenger ribonucleic acid during sporulation in *Bacillus subtilis*. J Biol Chem 1971;246:3189–95.
7. Carrera M, Zandomeni RO, Fitzgibbon J, Sagripanti J-L. Difference between the spore sizes of *Bacillus anthracis* and other *Bacillus* species. J Appl Microbiol 2006;102:303–12.
8. Fellows RF, Linscott MK, Ivins BE, Pitt MLM, Rossi CA, Gibbs PH, et al. Efficacy of a human anthrax vaccine in guinea pigs, rabbits, and rhesus macaques against challenge by *Bacillus anthracis* isolates of diverse geographical origin. Vaccine 2001;19:3241–7.
9. Dauphin LA, Newton BR, Rasmussen MV, Meyer RF, Bowen MD. Gamma irradiation can be used to inactivate *Bacillus anthracis* spores without compromising the sensitivity of diagnostic assays. Appl Environ Microbiol 2008;74(14):4427–33.
10. Scientific Working Group on Forensic Analysis of Chemical Terrorism (SWGFACT). Validation guidelines for laboratories performing forensic analysis of chemical terrorism. Forensic Science Communications 2005;7(2).
11. Seitz W, Schultz W, Weber WH. Novel applications of highly sensitive liquid chromatography/mass spectrometry/mass spectrometry for the direct detection of ultra-trace levels of contaminants in water. Rapid Commun Mass Spectrom 2006;20:2281–5.
12. Busetti F, Linge KL, Blythe JW, Heitz A. Rapid analysis of iodinated x-ray contrast media in secondary and tertiary treated wastewater by direct injection liquid chromatography-tandem mass spectrometry. J Chromatogr A 2008;1213:200–8.
13. Wahl KL, Colburn HA, Wunschel DS, Petersen CE, Jarman KH, Valentine NB. Residual agar determination in bacterial spores by electrospray ionization mass spectrometry. Anal Chem 2010;82:1200–6.

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