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Rapid Determination of Domoic Acid in Serum and Urine by Liquid Chromatography−Electrospray Tandem Mass Spectrometry

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A rapid, selective, and sensitive LC-MS/MS method was developed for the quantitative determination of domoic acid in serum and urine samples. Samples were prepared for analysis using an Oasis HLB SPE column. Determination was by a reversed phase HPLC using a mixture of methanol, acetonitrile, and water containing 1% acetic acid and an electrospray ionization (ESI) ion-trap mass spectrometer (Finnigan LCQ). The method was validated by analyzing five replicates each of negative control bovine serum or urine fortified with domoic acid at the 0.005 μg/g method detection limit (MDL) and at the 0.05 μg/g level. Recoveries ranged from 90 to 95% for fortifications at the MDL and from 92 to 98% for fortifications 10 times higher than the MDL. The diagnostic utility of the method was tested by analyzing samples from live animals showing clinical signs suggestive of domoic acid poisoning submitted to the veterinary toxicology laboratory.

KEYWORDS: Domoic acid; HPLC; mass spectrometry; LC/MS; urine; serum

INTRODUCTION

The global increase of phytoplankton blooms and their production of potentially harmful compounds has gained increased attention in the human, veterinary, and public safety communities around the world (*1*, *2*). Domoic acid is a neurotoxic amino acid (**Figure 1**) produced by several species of the diatom genus *Pseudo-nitzschia*, common in the marine plankton (*3*). Several cases of illness and deaths in humans, birds, and marine mammals have been attributed to domoic acid (*1*−*6*). After exposure, domoic acid binds predominantly to *N*-methyl-D-aspartate (NMDA) receptors in the central nervous system, causing depolarization of the neurons and excitotoxicity (*7*). Clinical signs resulting from domoic acid poisoning include gastrointestinal irritation (vomiting, diarrhea), ataxia, seizures, head waving, depression, and death. Kinetic studies in monkeys and rats have demonstrated that domoic acid is primarily excreted unchanged in urine following absorption (*8*−*10*).

The majority of analytical methods focus on the determination of domoic acid in shellfish, fish, and other vectoral material (*11*−*14*). However, it is virtually impossible to monitor all coastal waters, fish, and shellfish for potentially toxic phytoplankton blooms. Pertinent information about harmful algae may initially become available from affected marine mammals. In suspected cases of domoic acid poisoning, the gastrointestinal tract of animals is usually completely empty because of the direct emetic effect of domoic acid on the area postrema of the brain (*15*). Serum and urine are easily available in such cases, and the liquid chromatography−mass spectrometry (LC-MS) analysis described here provides rapid, unambiguous identification and quantitation of domoic acid. Positive results from biological specimens provide very important epidemiological information to intensify monitoring of fish and shellfish in areas where animals became ill, which will ultimately protect public health.

The existing methodology for quantitative determination of domoic acid in biological fluids (*16*, *17*) does not offer the necessary sensitivity or specificity needed to detect domoic acid at the parts per billion level. This emphasizes the need for rapid identification and sensitive quantitative determination of the toxin in urine and serum samples from live animals presented with clinical signs indicative of domoic acid poisoning. The LC−tandem mass spectrometry technique, previously employed for the analysis of shellfish for domoic acid (*11*), offers the extraordinary specificity and sensitivity necessary for unequivocal identification of the toxin; therefore, this technique was employed in the present study.

This paper describes a sensitive and specific analytical method for the screening and confirmation of domoic acid in urine and serum using high-performance liquid chromatography (HPLC) with positive ion electrospray tandem ion-trap mass spectrometry. The method offers both the MS/MS and MS/MS/MS identification of the toxin as well as quantification at the low parts per billion range. The method is designed for relatively quick turnaround of large sample sets. The diagnostic utility of the method has been tested by analyzing samples presented to the veterinary diagnostic laboratory.

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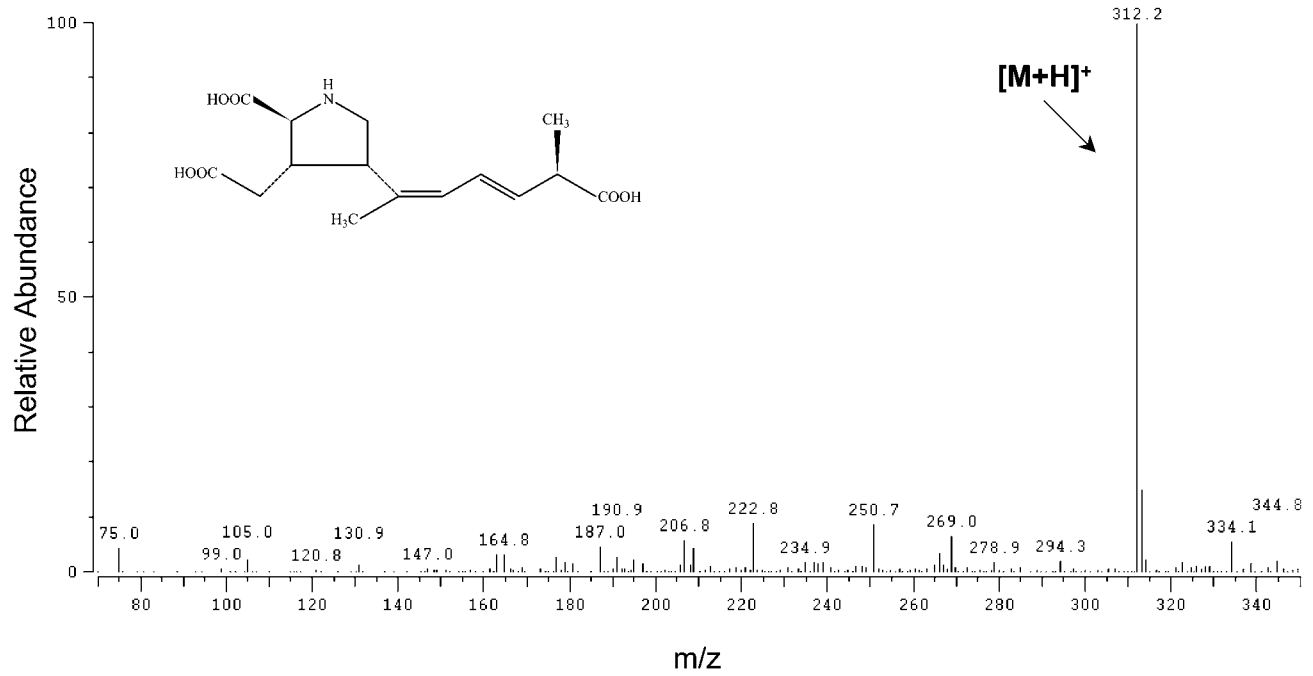

Figure 1. Positive ion ESI mass spectrum of domoic acid [CAS 14277-97-5], MW 311.3. Conditions: infusion (10 μL/min) of 10 μg/mL analytical standard into a mobile phase composed of 1% acetic acid in water (v/v, solution A), 1% acetic acid in methanol (v/v, solution B), and acetonitrile (solution C), run isocratically in proportion of 23% A, 57% B, 20% C (v/v/v) at 0.5 mL/min.

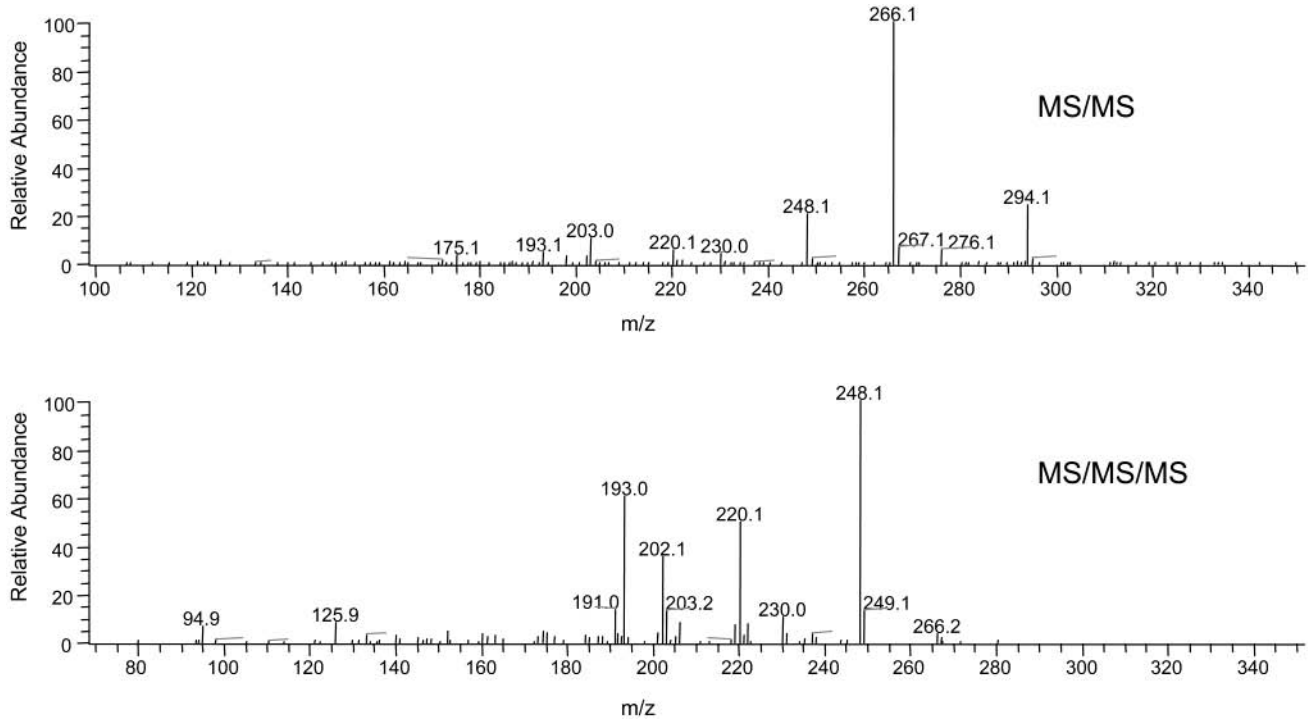

Figure 2. Positive ion ESI-MS/MS product ion spectrum (top) and MS/MS/MS product ion spectrum (bottom) of domoic acid in analytical standard. The MS/MS spectrum was obtained by isolating and fragmenting the protonated molecular ion of *m*/*z* 312. The MS/MS/MS spectrum was obtained by isolating and fragmenting of the MS/MS product ion of *m*/*z* 266.

Table 1. Average Recoveries (n = 5 Each) and Coefficients of Variation (% CV) of Domoic Acid from Fortified Bovine Urine and Serum Samples Analyzed by LC-MS/MS

fortification level, μg/g	urine % recovery (% CV)	serum % recovery (% CV)
0.05	98 (6.7)	92 (7.7)
0.005[a]	90 (4.5)	95 (14.7)

[a] Fortification at the method detection limit (MDL).

MATERIALS AND METHODS

Reagents. Water and acetonitrile were of HPLC grade (Fisher Scientific), and methanol was of HPLC grade (B&J Brand). Glacial acetic acid and orthophosphoric acid (85%) were of HPLC grade (Fisher Scientific). All HPLC running solvents were filtered through 0.45 μm nylon filters (Gelman Sciences, Ann Arbor, MI).

Preparation of Standard Solutions. Domoic acid standard was obtained from Sigma Chemical Co. (St. Louis, MO). Stock solution of 500 μg/mL was made in methanol. It was stable for 6 months when

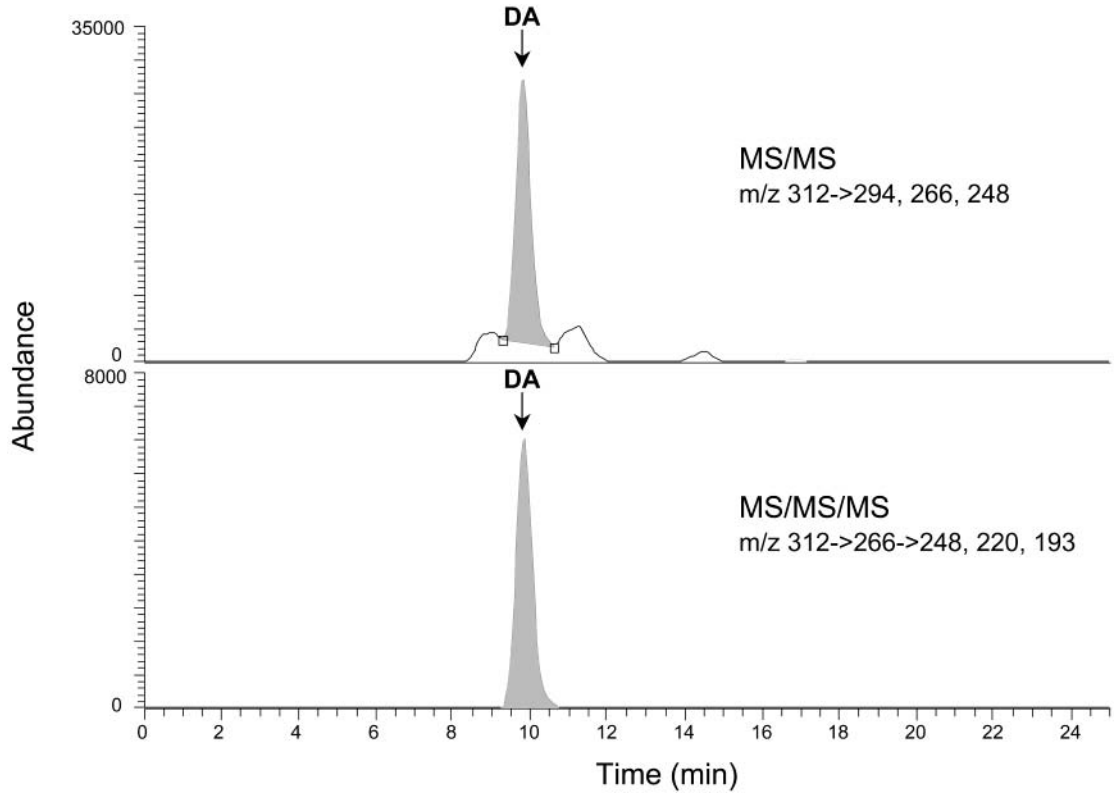

Figure 3. Typical LC-MS/MS (top) and LC-MS/MS/MS (bottom) chromatograms of control bovine serum fortified with domoic acid at a method detection limit of 0.005 μg/g. Plots of the sum of *m*/*z* 294, 266, and 248 from the MS/MS product ion spectrum of *m*/*z* 312 (top) were used for quantitation. Plots of the sum of *m*/*z* 248, 220, and 193 (bottom) from the MS/MS/MS product ion spectrum of *m*/*z* 266 (bottom) were used for confirmation. Ten microliters of 4 g/mL serum fortified with domoic acid at MDL level was injected. The retention time for domoic acid was 9.9 min.

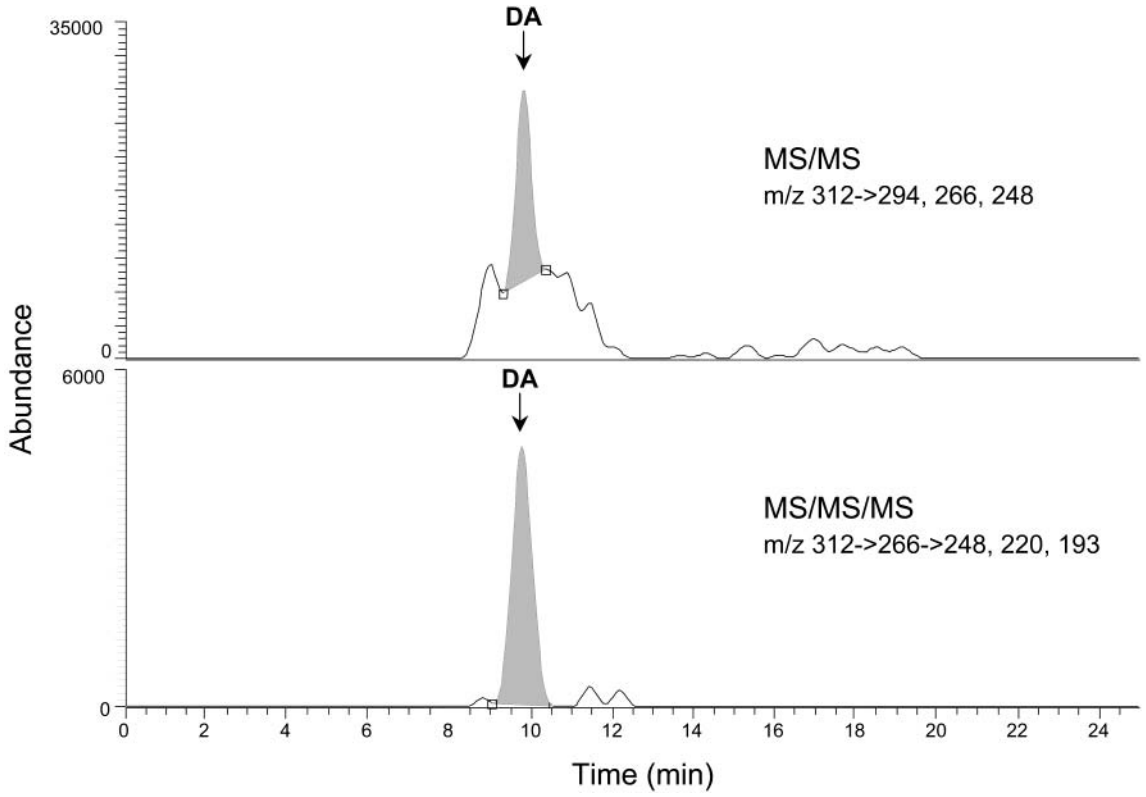

Figure 4. Typical LC-MS/MS (top) and LC-MS/MS/MS (bottom) chromatograms of control bovine urine fortified with domoic acid at a method detection limit (MDL) of 0.005 μg/g. Plots of the sum of *m*/*z* 294, 266, and 248 from the MS/MS product ion spectrum of *m*/*z* 312 (top) were used for quantitation. Plots of the sum of *m*/*z* 248, 220, and 193 (bottom) from the MS/MS/MS product ion spectrum of *m*/*z* 266 (bottom) were used for confirmation of positives. Ten microliters of 4 g/mL urine fortified with domoic acid at MDL level was injected. The retention time for domoic acid was 9.9 min.

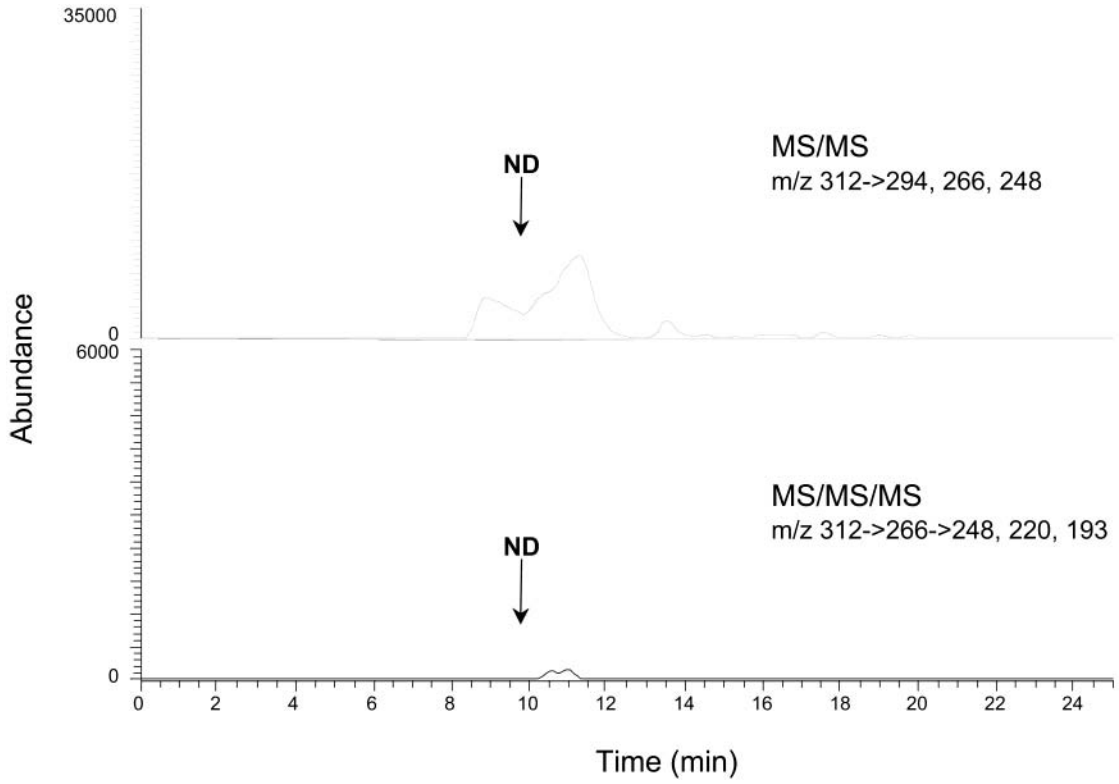

Figure 5. Typical LC-MS/MS (top) and LC-MS/MS/MS (bottom) chromatograms of control bovine urine. Plots of the sum of *m/z* 294, 266, and 248 from the MS/MS product ion spectrum of *m/z* 312 (top) were used for quantitation. Plots of the sum of *m/z* 248, 220, and 193 (bottom) from the MS/MS/MS product ion spectrum of *m/z* 266 (bottom) were used for confirmation of positives. Ten microliters of 4 g/mL control urine was injected. The retention time for domoic acid was 9.9 min.

stored at 4 °C. Subsequent dilutions of the standard were made daily in methanol from the stock solution. A four-point calibration curve in serum or urine was prepared by adding 2.5, 12.5, 50, and 125 μL of the 1 μg/mL daily working standard to dry, negative control serum or urine SPE eluants, prepared as described below, and adjusting the volume to 250 μL with methanol.

Sample Preparation. One gram of urine or serum sample was weighed into a 15 mL screw-cap, disposable tube. One milliliter of water and 20 μL of phosphoric acid were added to each sample followed by gentle mixing for 10 s. The mixture was quantitatively transferred onto an Oasis HLB extraction cartridge (60 mg, 3 cm^3, Waters Corp., Bedford, MA), prewashed with 3 mL of methanol and 3 mL of water. The column was washed with two 1 mL portions of 5% methanol in water (v/v) to remove interferences. Domoic acid was eluted from the column with 4 mL of 50% methanol in water (v/v) into a glass, disposable test tube. The eluate (extract) was evaporated to dryness, using a nitrogen evaporator (N-Evap, Analytical Evaporator, Organomation Associates Inc., Berlin, MA) set at 60 °C. The extract was redissolved in 250 μL of methanol and filtered through a 0.45 μm HPLC filter (Millipore Corp., Milford, MA) into a small-volume autosampler vial. All control and fortified samples were prepared in the same manner.

Liquid Chromatography—Mass Spectrometry. A Hewlett-Packard (HP) model 1050 high-performance liquid chromatograph coupled with a Finnigan LCQ ion-trap mass spectrometer was used in all analyses. The analytical column was a SphereClone ODS (*2*), 250 mm $\times$ 4.6 mm $\times$ 5 μm particle size (Phenomenex Inc., Torrance, CA), with a C18 guard column cartridge. The injection volume was 10 μL. The HPLC mobile phase was a mixture of 1% acetic acid in water (v/v, solution A), 1% acetic acid in methanol (v/v, solution B), and acetonitrile (solution C), run isocratically in proportion of 23% A, 57% B, and 20% C (v/v/v). The flow rate was 0.5 mL/min. The column effluent was diverted to waste for the first 8.5 min and during equilibration. The retention time for domoic acid was 9.9 min. MS data were acquired in the positive ion electrospray ionization (ESI) mode using a single segment with two alternating scan events. The maximum inject times were 200 ms. Scan event 1 (MS/MS) isolated *m/z* 312 with an isolation window of 3.0 amu, applied collision energy (CE) of 29.0%, and scanned the product ion spectrum from *m/z* 100 to 350. The response of the sum of ions *m/z* 294, 266, and 248 from scan event 1 was used for quantitation. Scan event 2 (MS/MS/MS) isolated and fragmented *m/z* 312 as above, then isolated the MS/MS product ion of *m/z* 266 (isolation window 2.5 amu, CE of 29.0%), and scanned the product ion spectrum from *m/z* 70 to 350. MS/MS/MS spectra were used for additional confimation of results. The electrospray interface was typically operated using the following settings: capillary voltage, 15 V; capillary temperature, 230 °C; spray voltage, 4.7 kV; tube lens voltage, 10 V. The instrument was tuned by optimization of *m/z* 312 while a 10 μg/mL domoic acid standard was infused at 10 μL/min into the mobile phase as above. Ten microliters of standards in matching matrix or sample extracts was injected into the system above. Each set of samples contained a reagent blank, control, and fortified samples. Quantification was by comparison with a four-point calibration curve using external standards in matching matrix and nonweighted second-order regression.

Method Validation. The method was validated by analyzing serum or urine samples ($n = 5$ each) fortified with domoic acid at the 0.005 μg/g method detection limit (MDL) and at the 0.05 μg/g level. The fortifications were prepared by adding 5 and 50 μL of 1 μg/mL domoic acid standard, respectively, to 1 g samples of negative, control bovine urine and serum and analyzing them using the method described above. The method was also tested by analyzing samples from diagnostic cases. Each set of diagnostic samples included a reagent blank, control samples, and fortified samples at the MDL and 10 times the MDL.

RESULTS AND DISCUSSION

Figure 1 shows the full-scan, positive ESI mass spectrum of domoic acid obtained during the infusion of domoic acid into the mobile phase as described. Protonation and sodium cluster formation was the main ionization process observed, with $[M + H]^+$ (*m/z* 312) as the base peak. The spectrum is consistent

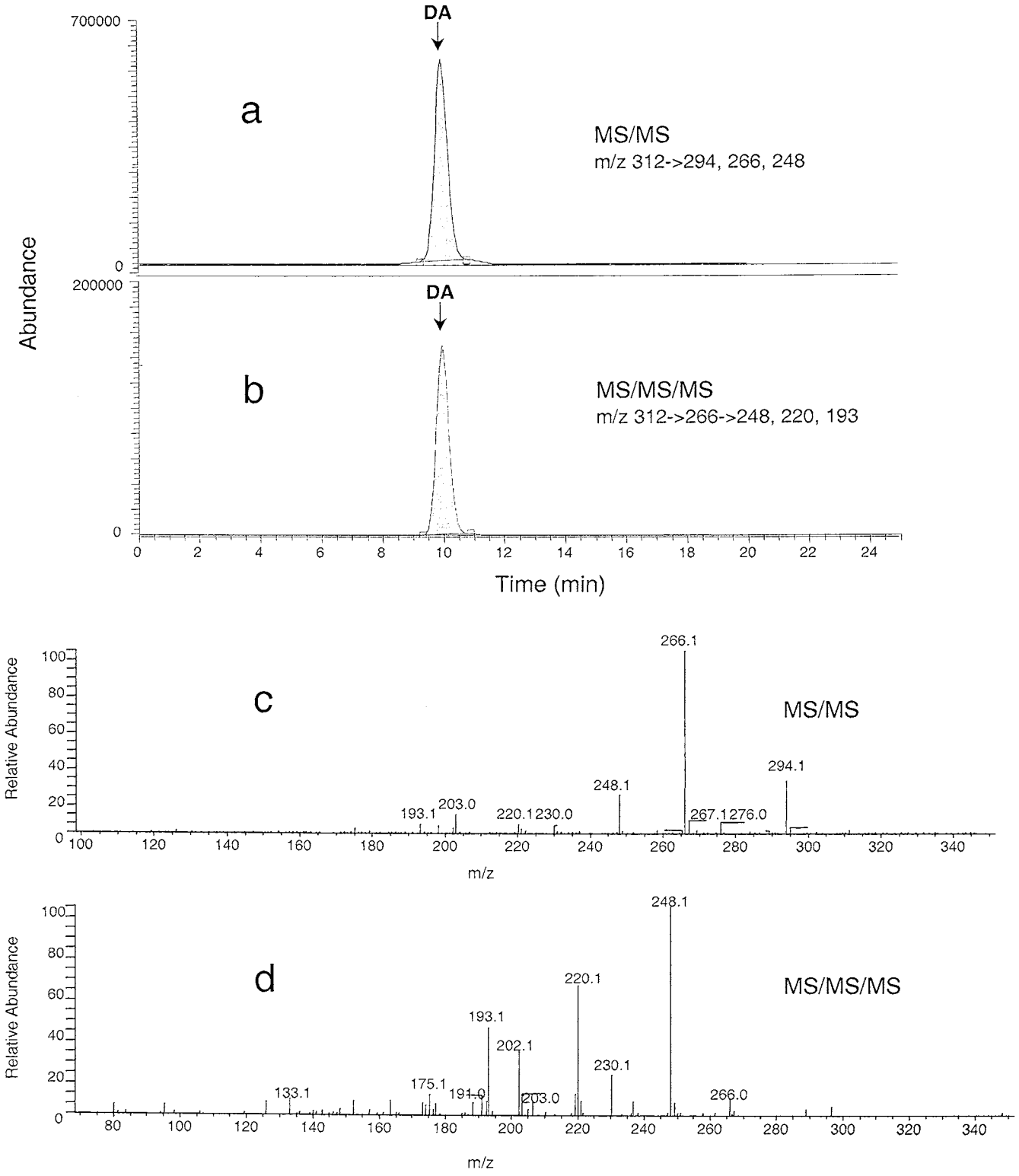

Figure 6. Typical LC-MS/MS (a) and LC-MS/MS/MS (b) chromatograms of domoic acid in a urine extract from a marine mammal. Plots of the sum of *m/z* 294, 266, 248 from the MS/MS product ion spectrum of *m/z* 312 (a) were used for quantitation. Plots of the sum of *m/z* 248, 220, 193 from the MS/MS/MS product ion spectrum of *m/z* 266 (b) were used for confirmation of positives. Ten microliters of 0.34 g/mL sample extract was injected. The concentration of domoic acid in urine was 1.403 μg/g. The retention time for domoic acid was 9.9 minutes. Positive ion ESI LC-MS/MS product ion spectrum (c) and LC-MS/MS/MS product ion spectrum (d) of domoic acid in a marine mammal urine sample (1.403 μg/g).

with the ESI spectrum previously reported (*11*). Three prominent product ions at *m/z* 294 $[M + H - H_2O]^+$, *m/z* 266 $[M + H - HCOOH]^+$, and *m/z* 248 (loss of two molecules of formic acid) were observed in the MS/MS spectrum under the conditions described. The sum of these ions was used for quantitation. The MS/MS/MS conditions were used for additional identification and confirmation of the positives. Confirmation was based on the ion ratios of the MS/MS/MS product ions (*m/z* 248, 220, and 193) in samples matching those in the standards and spikes of equivalent concentration within 20%. **Figure 2** shows typical MS/MS and MS/MS/MS product ion spectra of domoic acid in an analytical standard prepared in methanol.

The use of tandem mass spectrometry produced clean chromatograms of domoic acid with minimal background contribution from urine or serum matrix. The Oasis HLB SPE column removed matrix interferences and offered a quick, single-step sample preparation for analyses. Typical LC-MS/MS and LC-MS/MS/MS chromatograms of domoic acid in a control serum fortified at the MDL of 0.005 μg/g, control urine fortified at the MDL of 0.005 μg/g, and control negative urine are shown in **Figures 3−5**.

Domoic acid recoveries from spiked urine and serum samples are summarized in **Table 1**. Recoveries ranged from 90 to 95% for the fortifications at the MDL and from 92 to 98% for fortifications 10 times higher than the MDL. The coefficient of variation was highest (CV = 14.7%) when serum samples fortified at the MDL level were analyzed. This high variability was due to the instability of the detector's response to domoic acid, rather than the variability in sample preparation. Repeated injections of serum sample fortified at the MDL of 0.005 μg/g (n = 10 injections in a row) yielded a CV of 8.3%. Lower variability (CV = 1.3%) was observed for 10 injections of serum sample fortified at a higher, 0.05 μg/g, level. Quantitation of domoic acid in urine and serum using electrospray ionization was affected by ion suppression. This was accounted for by using standard curves in urine or serum. The standard curves in serum and urine followed second-order regression with r^2 values in the range of 0.9917−0.9986.

The described method was applied to the analysis of over 50 serum and urine samples from marine mammals. The range of domoic acid levels found in urine samples was 5−10000 ppb, whereas the serum levels ranged from 5 to 180 ppb. **Figure 6a**,**b** shows a typical chromatogram of a urine extract from a marine mammal, positive for domoic acid at 1.403 μg/g (1403 ppb). Domoic acid was clearly identified in the urine sample by comparing the MS/MS and MS/MS/MS product ion spectra to the spectrum of an analytical standard (**Figure 6c**,**d**).

This method is a significant improvement over the existing methodology in that it provides a rapid and unequivocal determination of domoic acid in biological fluids at very low, parts per billion levels. Batches of 10 samples can be easily extracted and analyzed by one analyst in a day. The sensitivity and specificity of the method enables it to be used as a diagnostic tool in domoic acid poisoning cases in live animals. This procedure was successfully used in the veterinary diagnostic laboratory to rapidly diagnose dietary exposure to domoic acid, especially in cases where stomach contents were not available for testing. The importance of domoic acid as a cause of poisoning may increase because of an increased frequency of harmful algal blooms. Future work will include expanding the method to provide additional testing for domoic acid in other matrices, such as urates and kidney.

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