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Determination of Strophanthidin in Ingesta and Plant Material by LC-MS/MS

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An LC-MS/MS method was developed for the semiquantitative determination of strophanthidin glycosides in ingesta from animals. Strophanthidin glycosides were simultaneously extracted and hydrolyzed to the strophanthidin aglycone using aqueous methanolic hydrochloric acid and the extracts cleaned up using solid-phase extraction. Extracts were analyzed using reverse-phase HPLC coupled with positive ion electrospray mass spectrometry. Characteristic product ion spectra were produced by fragmentation of the $[M + H]^+$ precursor ion for each analyte. Quantitation was performed using the internal standard method with digitoxigenin serving as the internal standard. The method detection limit was calculated to be 0.075 μg/g, and the limit of quantitation was calculated to be 0.24 μg/g for strophanthidin in control rumen samples. This method was used in diagnostic investigations to confirm fatal strophanthidin glycoside poisonings in horses.

KEYWORDS: Strophanthidin; cardiotoxic glycosides; HPLC; mass spectrometry; LC-MS

INTRODUCTION

Cardiotoxic glycosides are produced by a variety of plant and animal species and include compounds such as cymarin, digitoxin, digoxin, oleandrin, and bufotenin. These compounds share the common features of a steroidal aglycone linked at the 3β-OH group to one or more sugar moieties. Although some cardiac glycosides are therapeutically valuable and widely prescribed, they are also highly toxic to humans and animals (*1*). Cardiac glycosides inhibit Na^+,K^+-ATPase, resulting in a positive inotropic effect at therapeutic doses but in a variety of cardiac arrhythmias and death at toxic doses (*2*). In addition, cardiac glycosides act directly on the gastrointestinal tract, causing hemorrhagic enteritis and diarrhea. Strophanthidin ((3β,5β)-3,5,14-trihydroxy-19-oxocard-20(22)-enolide, **Figure 1**) is the aglycone of cardiac glycosides present in dogbane (*Apocynum* spp.), corkscrew flower (*Strophanthus speciosus*), silk vine (*Periploca graeca*), wallflower (*Erysimum* spp.), lily of-the-valley (*Convallaria majalis*), adonis (*Adonis* spp.), and tassa jute (*Corchorus olitorius*) (*3*). These compounds are considered quite toxic with an IV LD_{50} of 0.33 mg/kg in rats for strophanthidin and lower LD_{50} values for several of the glycosides (*4*−*6*).

Diagnostic methods for a variety of cardiac glycosides are well established. Digitalis glycosides have been analyzed in a variety of matrices by TLC (*7*−*9*), HPLC (*8*, *10*−*13*), micellar electrokinetic chromatography (*14*), and HPLC-MS/MS (*15*−*17*) as well as numerous immunoassays. Oleandrin has been analyzed by TLC (*9*, *18*), by HPLC with fluorescence detection (*19*−*20*), and by HPLC-MS/MS (*21*−*23*). Strophanthidin and digitoxigenin glycosides have been identified in *Corchorus olitorius* by hydrolysis of the glycosides and analysis of the resulting aglycones by HPLC (*24*, *25*). The present study was designed to develop a semiquantitative LC-MS/MS method for the determination of strophanthidin as the hydrolysis product of cardiac glycosides in gastrointestinal contents and plant material, with detection limits sufficient for diagnostic investigations and positive identification through tandem mass spectrometry.

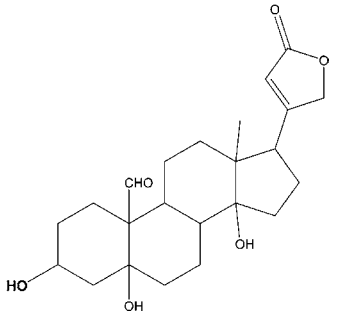

Figure 1. Chemical structure of strophanthidin. The 3β-OH (the point at which the sugar moieties attach) is identified in bold.

MATERIALS AND METHODS

Reagents. Methanol and glacial acetic acid (HPLC grade), hydrochloric acid, (HCl, Trace Metals grade), and concentrated ammonium hydroxide (NH_4OH, ACS Grade) were obtained from Fisher Scientific

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(Pittsburgh, PA). Water (18 MΩ) was obtained from a Barnstead Nanopure system. EIA buffer (1.0 M phosphate buffer, pH 7) was obtained from Neogen Corporation (Lexington, KY).

Preparation of Standard Solutions: Cymarin, strophanthidin, and digitoxigenin were purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions of 1.0 mg/mL of each compound were prepared in methanol. Dilutions of the stock standards were prepared at 100 μg/mL and 10 μg/mL for each compound in methanol.

Fortifications and Calibration Standards. Typical well-mixed bovine rumen samples were used as negative controls for ingesta analysis. Typical dried grass hay samples were used as negative controls for plant analysis. Samples of 20 and 50 μL of the 10-μg/mL strophanthidin standard solution were added to 1.0-g aliquots of control samples to produce strophanthidin fortified control samples at levels of 0.20 μg/g and 0.50 μg/g. A 20-μL aliquot of the 100-μg/mL strophanthidin standard solution was added to 1-g aliquots of control matrix to produce strophanthidin fortified control samples at a level of 2.0 μg/g. Cymarin fortified control samples were similarly prepared at a level of 0.50 μg/g by addition of 50 μL of the 10-μg/mL standard solution to 1-g aliquots of control matrix. Control rumen extracted as per the procedure detailed below was used for calibration standards. A 5-μL aliquot of each of the 1000-μg/mL standards of strophanthidin and cymarin were added to a 1-mL volume of control rumen extract to produce a mixed calibration standard at 5.0 μg/mL. Samples of 10 and 20 μL each of 10-μg/mL strophanthidin and cymarin standards were added to 1-mL volumes of control rumen extract to produce mixed calibration standards at 1.0 and 2.0 μg/mL of each analyte. The 5.0 μg/mL standard was then diluted 1:10, 1:50, and 1:100 in control rumen extract to produce mixed calibration standards of 0.50, 0.10, and 0.050 μg/mL. A 5.0-μL aliquot of a solution containing 1000 μg/mL of digitoxigenin was added to each 1.0-mL standard aliquot. Standard solutions were stored at −4 °C. The 1000-μg/mL solutions were stable through the course of this study (approximately 6 months). Fortification and calibration solutions were stable for 4 week periods.

Extraction, Hydrolysis, and Cleanup Procedure. *Extraction and Hydrolysis.* Each batch of ingesta samples included one or more unfortified rumen control samples, one or more samples of control rumen fortified with strophanthidin, and one or more samples of control rumen fortified with cymarin. Each batch of plant samples included one or more unfortified grass hay control samples, one or more samples of control hay fortified with strophanthidin, and one or more samples of control hay fortified with cymarin. Samples (1 g) of ingesta or plant material were weighed into 16- × 125-mm test tubes, and 5 mL of 50% methanol in 3% aqueous HCl (v/v) was added. Samples were vortex mixed and placed in a 25 °C water bath for 3 h. The extracts were centrifuged at 1600 rpm (250*g*) for 10 min in an IEC CU-5000 centrifuge (International Equipment Co.). Aliquots (2.5 mL) of supernatant were transferred to 16- × 100-mm test tubes, 1 mL of EIA buffer was added, and samples were neutralized to pH 7 with ammonium hydroxide. The test tubes were then placed in a Turbo-Vap evaporator (Model LV, Zymark Corp.) set at 50 °C for 10 min with the nitrogen pressure set at 15 psig to reduce the methanol content in the extract prior to SPE cleanup. Approximately 3 mL of extract remained after evaporation.

Cleanup and Volume Reduction. Sep-Pak Vac C18 reverse-phase SPE cartridges of 3-cc, 500-mg capacity (Waters Corp) were preconditioned with 2 mL of methanol followed by 2 mL of water. Extracts were applied to the cartridges and allowed to flow through with minimal vacuum at approximately 1 mL/minute. Cartridges were rinsed sequentially with 2 mL of water and 2 mL of 20% methanol in water (v/v), and the analyte was eluted with 3 mL of methanol. The eluate was evaporated to dryness on the Turbo-vap at 50 °C and reconstituted with 0.5 mL of 50% methanol in water (v/v), giving a final concentration of 1 g/mL. A 5-μL aliquot of 1000 μg/mL digitoxigenin was added as an internal standard, and the samples were filtered through 0.45-μm × 4-mm PVDF syringe filters (Millipore Corp).

LC-MS/MS Analysis. A Hewlett-Packard 1050 HPLC coupled with a Finnigan LCQ ion trap mass spectrometer was used for all analyses.

HPLC Conditions. The HPLC column was a 150- × 4.6-mm. i.d., 5-μm, Luna C18(2) (Phenomenex Corp.). The column was maintained at ambient temperature. The mobile phase consisted of 1% acetic acid in water (v/v) (solvent A) and 1% acetic acid in methanol (v/v) (solvent B). The following gradient was used: 50% A for 1 min at 0.5 mL/min at injection, linear decrease to 10% A at 8 min at 0.5 mL/min, hold for 2 min. At 10 min, increase flow to 0.7 mL/min and switch to 50% A. The column was allowed to reequilibrate for 9 min at 0.7 mL/min of 50% A before another injection was made. Total analysis time (including reequilibration) was 19 min. Retention times for each analyte were as follows: strophanthidin, 8.4 min; cymarin, 10 min; digitoxigenin, 11.1 min.

Mass Spectrometer Conditions. Positive ion electrospray ionization (ESI) was used for all analyses. Mass calibration was performed as per the manufacturer's recommended procedures, using caffeine, L-methionyl-arginyl-phenylalanyl-alanine acetate, and Ultramark 1621. Optimization was performed by infusing a 10-μg/mL solution of strophanthidin standard directly into the mass spectrometer and using the instrument's automated tuning procedures. Solutions of cymarin and digitoxigenin (10 μg/mL) were infused for the purpose of establishing optimal collision energy settings for those compounds. Two acquisition segments were used. In segment 1 (0−9.3 min), the $[M + H]^+$ ion of strophanthidin at *m*/*z* 405 was isolated with a scan window of 2.0 and fragmented using a collision energy of 30%. The product ion spectrum from *m*/*z* 300−500 was acquired. Two separate scan events occurred in segment 2 (9.3−13 min). In the first scan event, the $[M + H]^+$ ion for digitoxigenin was isolated with a scan window of 2.0 and fragmented using a collision energy of 26%, and the product ion spectrum was acquired from *m*/*z* 115−470. In the second scan event, the $[M + H]^+$ ion for cymarin was isolated with a scan window of 2.0 and fragmented with a collision energy of 16%, and the product ion spectrum was acquired for *m*/*z* 150−580. A single tune file based on strophanthidin infusion was used for both acquisition segments. Injection volumes of 10 μL were used for samples and standards.

Data Evaluation. Qualitative identification in samples was by comparison of MS/MS spectra and LC retention times with standards. Gaussian smoothing (11-point, as provided by the instrument software) was used for chromatographic displays. Samples were considered positive for strophanthidin when a peak was detected within ± 0.2 min of the strophanthidin peak in the associated 0.5 μg/mL standard analysis and when the ions of *m*/*z* 341, 351, 359, 369, and 387 were within 20% of their relative peak heights from that same standard analysis. Quantitation was performed using digitoxigenin as the internal standard. Summed peak areas for product ions of *m*/*z* 341, 351, 359, 369, and 387 were integrated for strophanthidin; *m*/*z* 359, 369, 387, and 405 were integrated for cymarin; and the single product ion at *m*/*z* 339 was integrated for the digitoxigenin internal standard.

RESULTS AND DISCUSSION

This method provided semiquantitative data for strophanthidin in stomach content, cecum content, and colon content.

Positive ion ESI of strophanthidin gave the $[M + H]^+$ at *m*/*z* 405. MS/MS fragmentation of the ion at *m*/*z* 405 gave a distinctive product ion spectrum (**Figure 2**). Strophanthidin was routinely detected at a level of 0.50 ng, on-column (**Figure 2**). Relative response curves for strophanthidin followed first-order linear regression ($r^2 > 0.985$) in the 0.050−5.0 μg/mL range. Analysis of control rumen samples gave chromatograms free of interferences in the strophanthidin region (**Figure 3**).

Cymarin was used as a model compound to test hydrolysis efficiency. Hydrolysis efficiency, as measured by the analysis of control matrix fortified with cymarin, ranged from 40 to 90% through the course of the study. The effect of temperature upon hydrolysis and extraction was evaluated by analysis of cymarin-fortified rumen samples hydrolyzed at temperatures ranging from 25 °C to 70 °C. Hydrolysis and extraction at temperatures in the range of 25−30 °C gave the highest strophanthidin recoveries.

Replicate samples of rumen fortified with strophanthidin showed a coefficient of variation (CV) of 17% at 0.20 μg/g (mean recovery = 71%, $n = 7$) and 11% CV at 2.0 μg/g (mean

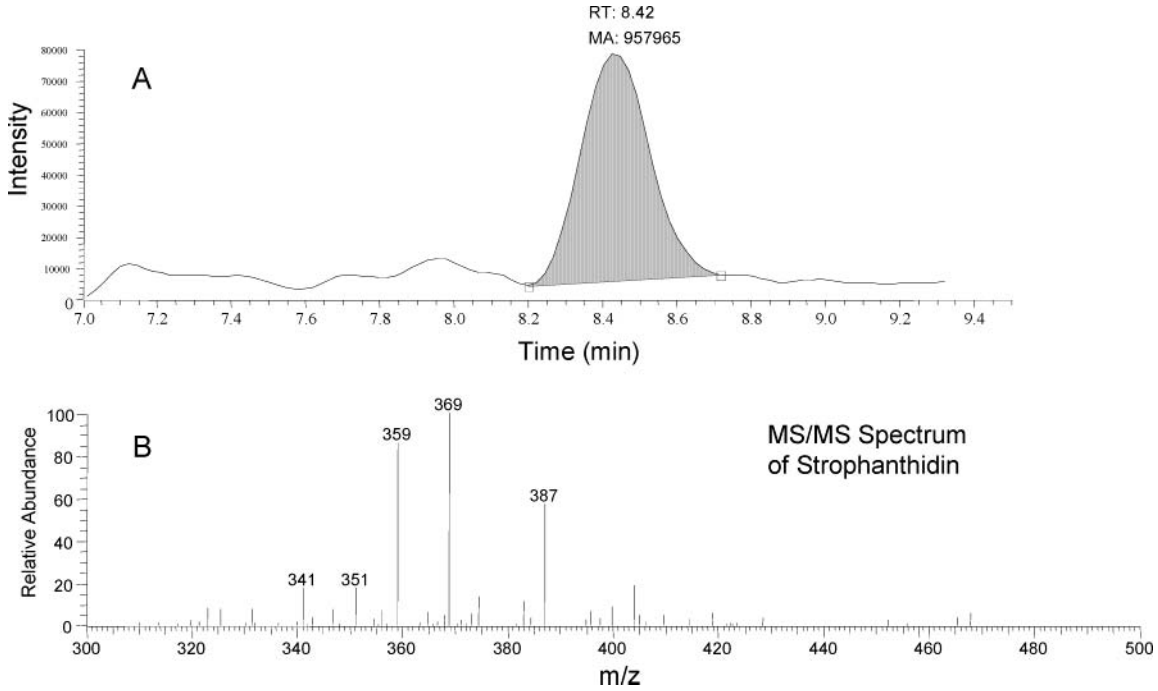

Figure 2. (**A**) LC-MS/MS chromatogram of a 0.05 μg/mL strophanthidin analytical standard (0.50 ng on-column). The chromatogram contains the plot of the sum of *m/z* 341, 351, 359, 369, and 387 from the MS/MS product ion spectrum of *m/z* 405. (**B**) Mass spectrum of the strophanthidin peak produced by isolation and fragmentation of the protonated molecular ion at *m/z* 405.

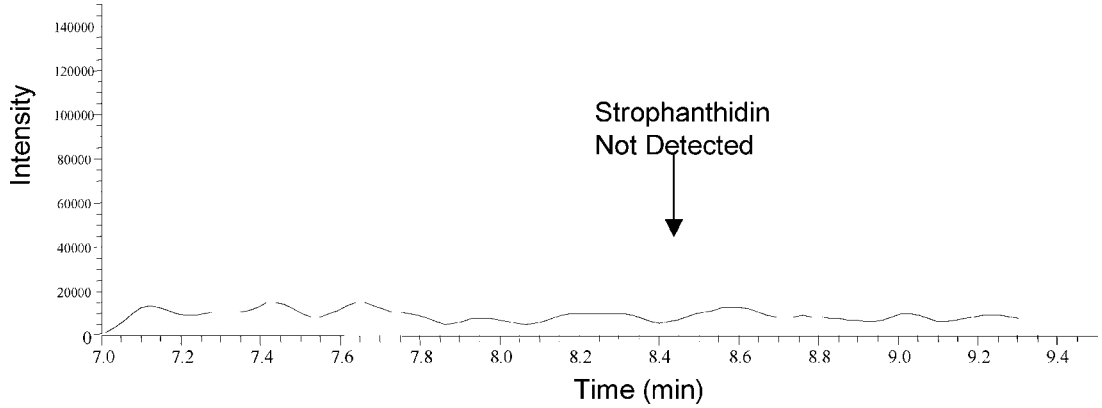

Figure 3. LC-MS/MS chromatogram of the retention time region for strophanthidin in unfortified control rumen showing no detectible strophanthidin.

recovery = 94%, $n = 5$). **Figure 4** shows a chromatogram from the analysis of one of these rumen samples fortified at 0.20 μg/g. The method detection limit at the 99% confidence level (as calculated by 3.14 × σ_{n-1} for 7 replicates fortified at 0.20 μg/g) (*26*) was 0.075 μg/g, and the limit of quantitation (10 × σ_{n-1} for 7 replicates at the same level) was calculated to be 0.24 μg/g. These data were sufficient for veterinary diagnostic analysis.

Strophanthidin detection is of excellent diagnostic use in suspect poisoning cases, as the pharmacological activity of the glycosides resides in the aglycone (*27*). Although the existence of strophanthidin glycosides in *Adonis* has been demonstrated (*28, 29*), structures have been described for only some of the cardenolides present, and very few of these are available commercially as analytical standards. The hydrolysis technique described here allows for detection of the toxic components without requiring identification of the individual glycosides or their synthesis or purification for use as analytical standards. The hydrolysis of all of the glycosides to a single product and analysis for that product was also advantageous in that it effectively increased the sensitivity of the method. This method could be used to obtain diagnostic information regarding suspect poisoning by any plant that contains strophanthidin glycosides. The ability to detect strophanthidin in biological specimens will prompt additional diagnostic investigations such as the identification of strophanthidin glycoside containing plants in the environment.

Because the efficiency of hydrolysis in any given sample could not be measured, we considered this assay to be semiquantitative. Although analysis for the hydrolyzed aglycone compromised the quantitative aspects of the resulting information, we felt that it offered the best technique currently available to support diagnoses of *A. aestivalis* poisoning in animals.

This method was used for diagnostic samples. Three horses submitted to the California Animal Health and Food Safety Laboratory for necropsy exhibited symptoms consistent with exposure to cardiac glycosides prior to their deaths. Necropsy of the horses showed distinct cardiac lesions, suggestive of exposure to cardiotoxic plants (*30*). Initial testing of stomach content from the affected horses showed no detectable levels of digitoxin, digoxin, grayanotoxins, or oleandrin. Inspection of grass hay fed to the horses shortly prior to their deaths

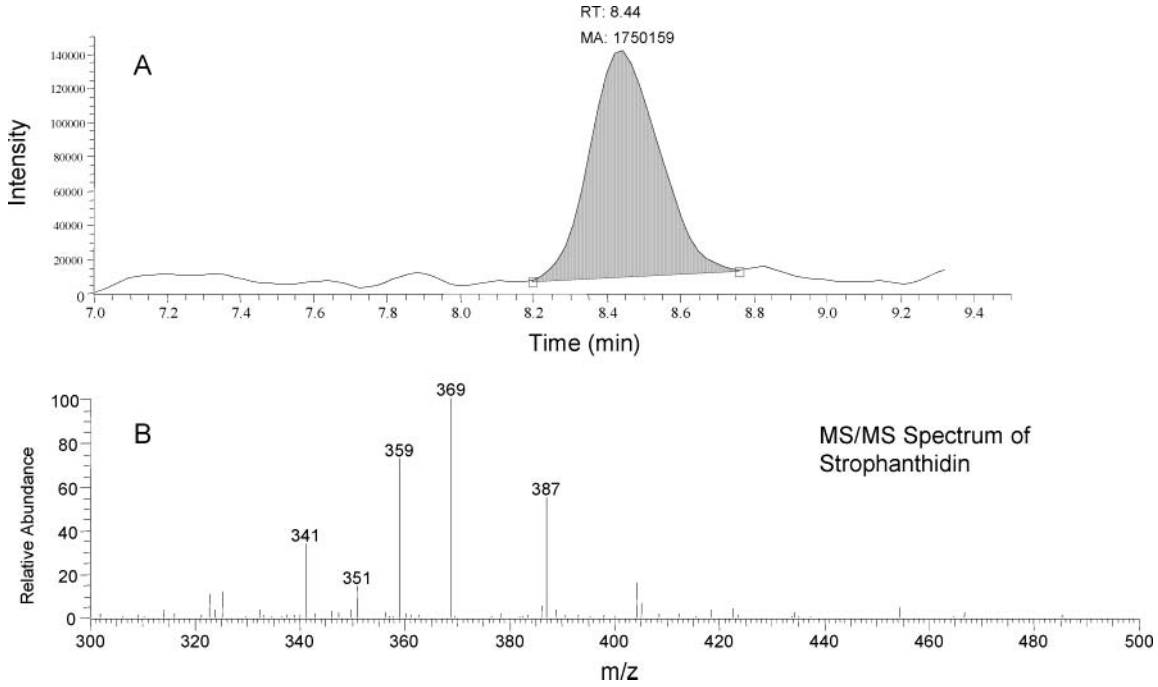

Figure 4. (**A**) LC-MS/MS chromatogram of control bovine rumen fortified with strophanthidin at a level of 0.20 ppm prior to extraction and analysis. (**B**) The mass spectrum taken from the peak at 8.44 min retention time.

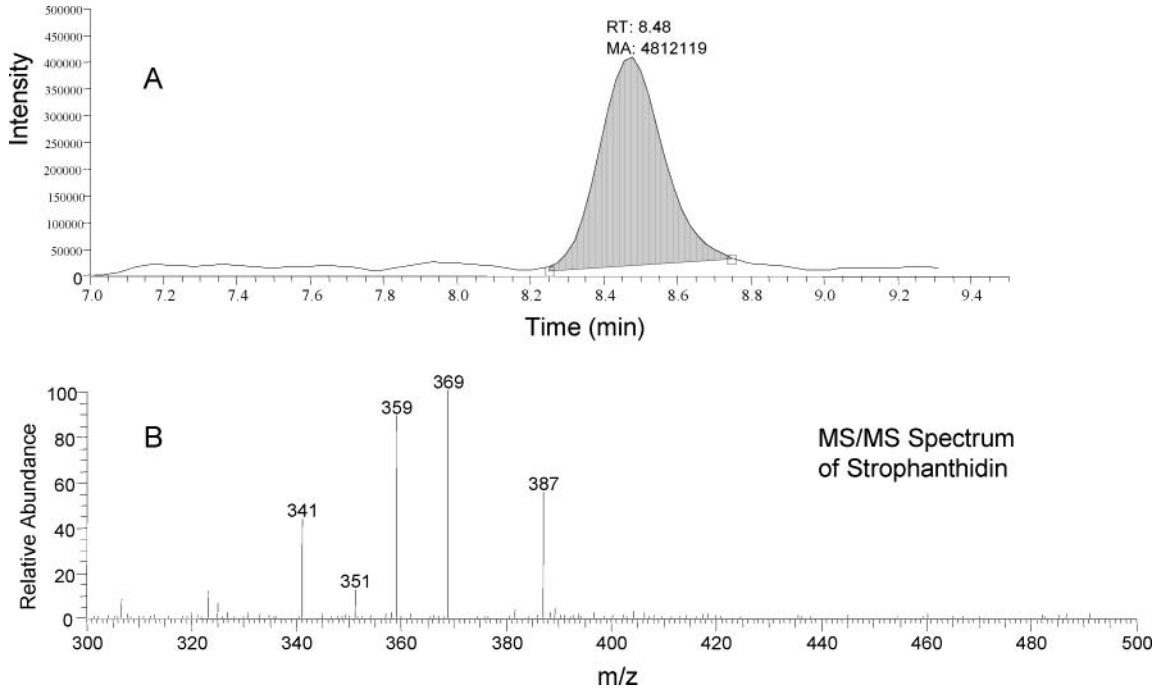

Figure 5. (**A**) LC-MS/MS chromatogram of strophanthidin in a sample of stomach content taken from a deceased horse suspected of having consumed hay contaminated with *Adonis aestivalis*. The level of strophanthidin in the stomach content was calculated to be 0.50 μg/g. (**B**) The mass spectrum from the peak at 8.48 min retention time, confirming the identification of strophanthidin.

revealed the presence of small amounts of dried plant material identified as *A. aestivalis*, or Summer Adonis. Strophanthidin was identified in the suspect plant material at a level of approximately 11 μg/g after hydrolysis. Subsequent analyses of stomach, cecum, and colon contents confirmed the presence of strophanthidin in all three horses at levels ranging from 0.5 to 0.8 μg/g after hydrolysis. A chromatogram and mass spectrum from one of these samples is shown in **Figure 5**. After these cases, we have analyzed stomach, cecum, intestinal, colon, and/or rumen samples from 15 animals suspected of having been exposed to cardiotoxic plants. Strophanthidin was detected in the intestinal content of one of these animals.

Combined hydrolysis and extraction of ingesta samples with subsequent analysis by LC-MS/MS can provide rapid evidence of exposure to plants containing strophanthidin glycosides. This method provides unambiguous identification of strophanthidin and a semiquantitative determination of the strophanthidin released from glycosides via hydrolysis. Sensitivity is sufficient to provide clinically relevant information. This method has provided information crucial in the diagnosis of strophanthidin

poisoning in horses The procedure is especially suited to veterinary diagnostic laboratory situations for which rapid diagnosis of exposure to cardiotoxic plants is necessary.

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