Determination of Grayanotoxins in Biological Samples by LC-MS/MS

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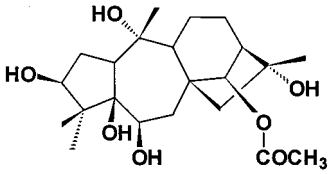
A rapid LC-MS/MS method was developed for the quantitative determination of grayanotoxins I, II, and III in rumen contents, feces, and urine. The grayanotoxins were extracted from solid samples with methanol. The methanol extract was diluted with water and cleaned up using a reversed phase solid phase extraction column. HPLC separation was performed by reversed phase HPLC using a gradient of water and methanol containing 1% acetic acid. Determination was by positive ion electrospray ionization and ion trap tandem mass spectrometry. Grayanotoxin I quantitation was based on fragmentation of the sodium adduct ion at *m*/*z* 435 to a product ion at *m*/*z* 375. Grayanotoxins II and III were quantitated on the basis of fragmentation of the ion at *m*/*z* 335 to the product ion at *m*/*z* 299. The method detection limits were 0.2 μg/g in rumen contents and feces and 0.05 μg/g in urine. Fortifications at the detection limits and 10 times the detection limits of bovine rumen contents, caprine feces, and ovine urine were recovered in the range 80−114%. The diagnostic utility of the method was tested by analyzing samples submitted to the veterinary toxicology laboratory.

Keywords: *Grayanotoxin; HPLC; mass spectrometry; LC-MS*

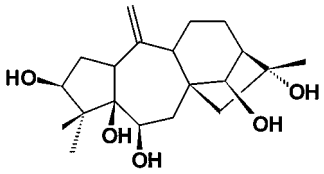
INTRODUCTION

Cardiotoxicity in animals can be caused by ingestion of several members of the Ericaceae (heath) family, including *Kalmia latifolia* (mountain laurel), *Rhododendron* spp. (rhododendrons and azaleas), and *Pieris* spp. (*1*, *2*). These species contain diterpene polyalcohols called grayanotoxins (Figure 1; *1*, *3*−*5*). Over 30 grayanotoxin-related compounds have been characterized (*3*), all having a tetracyclic A-*nor*-B-*homo*-*ent*-kaurane (andromedane) skeleton. Grayanotoxins I, II, and IV are the most toxic, with the i.p. LD_{50} in mice ranging from 0.87 to 1.3 mg/kg (*3*). Intoxication is indicated by bradycardia, hypotension, cardiac arrhythmias, gastrointestinal pain and spasm, muscular weakness, and paralysis (*1*, *6*, *7*). Grayanotoxins exert their toxic effects through an increase in the permeability of sodium ions in excitable membranes (*8*).

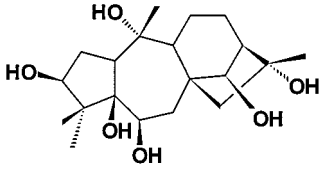
Grayanotoxins have been analyzed in plant material and honey by thin-layer chromatography (TLC) (*9*−*12*) and by direct spectrometric methods (*13*). Gas chromatography (GC) has been used to analyze the trimethylsilyl derivatives of grayanotoxins (*14*). A two-dimensional TLC method for the qualitative determination of grayanotoxins in animal ingesta and feces was able to detect grayanotoxins at 0.2 μg/g (*15*); however, this method was qualitative, and identification was based on a nonspecific colorometric reaction. Leaf concentrations of grayanotoxins are in the 100 μg/g range (*16*), so a method testing ingesta, urine, or feces to diagnose dietary exposure to grayanotoxin-containing plants should be quantitative at <1 μg/g. The present paper describes a quantitative LC-MS/MS method for the determination of grayanotoxins in feces, ingesta, and urine, with method detection limits in the nanograms per gram range and positive identification through tandem mass spectrometry.



GRAYANOTOXIN I



GRAYANOTOXIN II



GRAYANOTOXIN III

Figure 1. Chemical structures of grayanotoxins I, II, and III.

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10.1021/jf000750s CCC: $20.00
Published on Web 02/07/2001

MATERIALS AND METHODS

Reagents. Water, methanol, and acetic acid were of HPLC grade (Fisher Scientific).

Preparation of Standard Solutions. Pure grayanotoxins I, II, and III (GTX I, II, and III) were kindly provided by Dr. W. H. Tallent (U.S. Department of Agriculture, Beltsville, MD). Stock solutions of 1000 μg/mL were made in methanol. Mixed standards containing all three grayanotoxins at 10 μg/mL were made by dilution with methanol from the stock solutions and were stable for 6 months when stored at 5 °C. Dilute standards were prepared daily from the 10 μg/mL standard.

Fortifications and Calibration Standards. Typical well-mixed rumen content, feces, and urine samples that had been frozen and later thawed were used for negative controls. Grayanotoxin-fortified control samples were prepared at 2 and 0.2 μg/g by the addition of 50 μL of GTX mixed standard at 10 and 1.0 μg/mL, respectively. The samples were quickly mixed and then extracted. Standards in negative control feces, rumen content, and urine were prepared by the addition of mixed standard to the extract following the C-18 SPE cleanup. Standards in rumen content and feces extracts were prepared at 0.08, 0.032, 0.008, and 0.004 μg/mL. Standards in urine extract were prepared at 0.02, 0.01, 0.002, and 0.001 μg/mL.

Extraction and Cleanup Procedure. *(a) Extraction.* Well-mixed solid samples (0.25 g) were weighed into a 20 mL screw-capped test tube, 2.5 mL of methanol was added, and the sample was mixed for 30 min on a rotary mixer. The extract was centrifuged at 2000 rpm (260*g*) for 5 min using an IEC Centra-7 centrifuge (International Equipment Co.). An aliquot (1 mL) was taken, 5 mL of deionized water was added, and the extract was applied to a C-18 SPE column.

(b) C-18 SPE Cleanup. C-18 SPE columns, Oasis HLB 3 cm^3 60 mg extraction cartridges (Waters Corp., Milford MA), were prewashed with 3 mL of methanol followed by 3 mL of water/methanol (3:1). Extracts of feces and rumen content were loaded onto the columns without vacuum. Urine samples (1 g) were added directly to the column. The column was washed with 2 mL of water/methanol (3:1), and the grayanotoxins were eluted with 2.5 mL of water/methanol (1:4). This eluate was analyzed by LC-MS/MS.

LC-MS/MS Analysis. A Hewlett-Packard model 1050 HPLC coupled with a Finnigan LCQ ion trap mass spectrometer was used for all analyses. The analytical column was a Symmetry C-18, 15 cm × 4.6 mm × 5 μm particle size (Waters Corp.). Column temperature was ambient. The grayanotoxins were eluted under gradient conditions with 1% acetic acid in water (A) and 1% acetic acid in methanol (B) as follows: 70% A/30% B for 1 min, linear gradient to 10% A/90% B at 15 min, hold for 3 min. Flow rate was 0.5 mL/min.

MS data were acquired in the positive ion ESI mode using two alternating MS/MS scan events. Scan event 1 (for GTX II and GTX III) isolated *m/z* 335 with an isolation window of 3.0 amu and a collision energy of 14.5% and scanned the product ion spectrum from *m/z* 90 to 350. Scan event 2 (for GTX I) isolated *m/z* 435.2 with an isolation window of 1.5 amu and a collision energy of 16% and scanned the product ion spectrum from 120 to 500 amu. The instrument was tuned by optimizing the response of *m/z* 435.2 while infusing 10 μg/mL GTX III at 50 μL/min into a mobile phase of 1% acetic acid in water/methanol (1:1) at 0.5 mL/min.

Sample extract or standard (20 μL) was injected onto the LC-MS system. Quantification was by comparison with a four-point calibration curve using peak areas of external standards in matching matrix and second-order regression. The response of the ion at *m/z* 299 from scan event 1 was used for quantitation of GTX II and GTX III, whereas the response of the ion at *m/z* 375 from scan event 2 was used for quantitation of GTX I.

Method Validation. The method was validated by analyzing four replicates each of pooled negative control bovine rumen content, caprine feces, and ovine urine fortified at the method detection limits and 10 times the method detection limits.

Table 1. Summary of Validation Study Results[a]

forticication level (μg/g)	% recovery (% CV)		
	GTX I	GTX II	GTX III
ovine urine			
0.5	100 (11)	97 (16)	92 (14)
0.05	114 (3.1)	102 (13)	113 (11)
bovine rumen content			
2	95 (6.8)	91 (6.2)	88 (4.2)
0.2	92 (9.2)	97 (11)	81 (12)
caprine feces			
2	96 (12)	82 (7.6)	80 (12)
0.2	114 (18)	84 (8.7)	80 (11)

[a] Average percent recovery and percent coefficient of variation for sample fortifications ($n = 4$) with GTX I, II, and III in ovine urine, bovine rumen content, and caprine feces at the method detection limit (MDL) and 10 times the MDL.

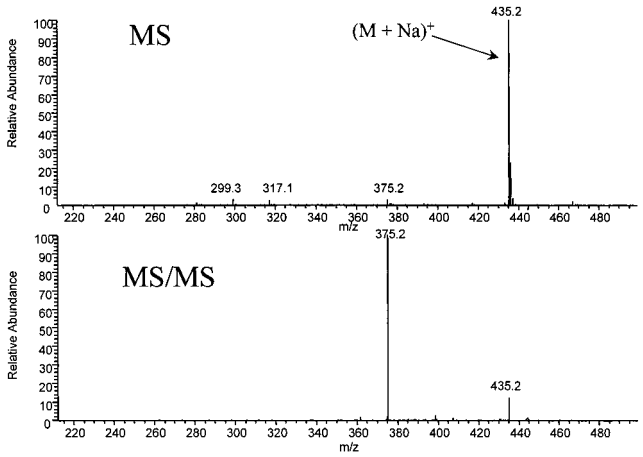

Figure 2. Positive ion ESI mass spectrum of GTX I, and the MS/MS spectrum from ion trap CID of the ion at *m/z* 435.2.

RESULTS AND DISCUSSION

The method quantitatively extracted and recovered all of the grayanotoxins in all three matrices studied (Table 1). Recoveries ranged from 80 to 114% for the detection limit fortifications and from 80 to 100% for fortifications at 10 times the method detection limits. The method variability ranged from 3.1 to 18% coefficient of variation (CV). High variability was mostly due to instability in detector response. Repeated analyses of ovine urine fortified at 2 μg/g ($n = 5$) yielded up to 10% CV. This level of precision was sufficient for veterinary diagnostic analyses. Method detection limits were based on concentrations that routinely gave a 10:1 signal to noise ratio and >70% recovery of fortified samples.

MS/MS detection enabled the use of a rapid method with minimal cleanup and no evaporative steps. Use of additional cleanups such as charcoal (*15*) resulted in variable and low (<70%) fortification recoveries. GTX I produced the sodium adduct ion under the positive ion electrospray conditions and fragmented to only one significant ion under the MS/MS parameters (Figure 2). Fresh standards gave the same proportion of sodium adduct as standards older than 1 month, indicating that the sodium adduct was formed from ubiquitous sodium in the HPLC system and not from sodium in the glass of the standard container. GTX II and GTX III electrospray spectra were characterized by some degree of fragmentation from successive losses of water, with the ion at *m/z* 335 the base peak for both compounds. The ion at *m/z* 335 was used as the precursor ion for GTX II

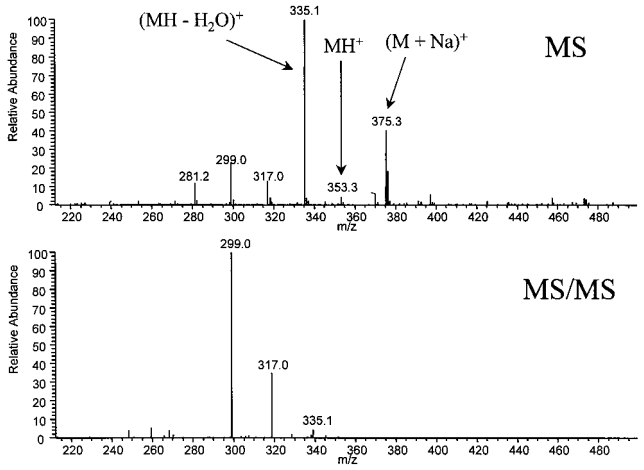

Figure 3. Positive ion ESI mass spectrum of GTX II, and the MS/MS spectrum from ion trap CID of the ion at *m*/*z* 335.1.

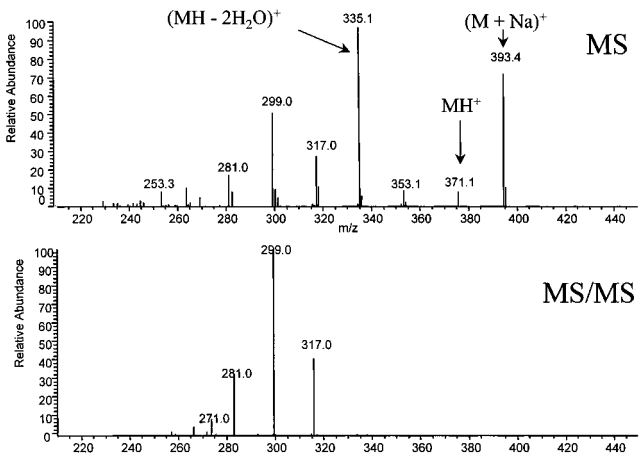

Figure 4. Positive ion ESI mass spectrum of GTX III, and the MS/MS spectrum from ion trap CID of the ion at *m*/*z* 335.1.

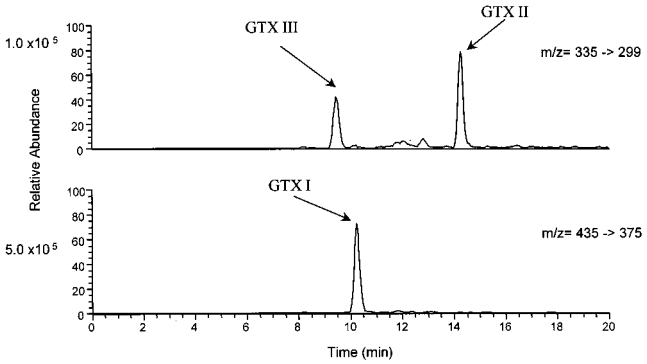

Figure 5. LC-MS/MS chromatogram of extract of ovine urine fortified at 0.5 μg/g with GTX I, II, and III. Twenty microliters of 0.4 g/mL sample extract was used. Plots of the ion trap CID transition of *m*/*z* 335 to 299 (top) and of *m*/*z* 435 to 375 (bottom) are shown.

and GTX III, producing similar product ion spectra (Figures 3 and 4).

The use of this rapid method, coupled with the use of MS/MS detection, resulted in very clean chromatograms at the concentrations tested (Figures 5−7). When a 2 μg/g urine fortification was concentrated 5-fold and injected, only a 2-fold increase in response was observed, indicating that matrix ion suppression was significant in more concentrated samples. Rumen content extracts produced similar ion suppression. The use of standards in matching matrix reduced the effects of ion suppression from matrix coextractives on quantitation. Although use of APCI may have reduced the effect of matrix on response, it did not provide adequate sensitivity. Because the sample extracts were in water/methanol, the addition of a concentration step to the method was undesirable.

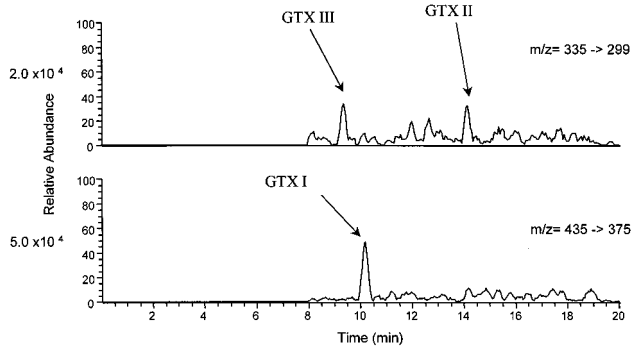

Figure 6. LC-MS/MS chromatogram of extract of ovine urine fortified at 0.05 μg/g with GTX I, II, and III. Twenty microliters of 0.4 g/mL sample extract was used. Plots of the ion trap CID transition of *m*/*z* 335 to 299 (top) and of *m*/*z* 435 to 375 (bottom) are shown.

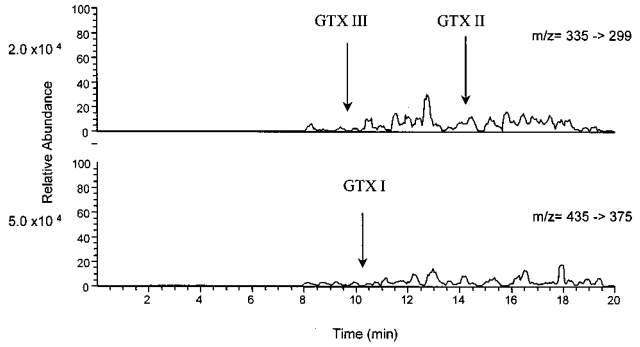

Figure 7. LC-MS/MS chromatogram of extract of negative control ovine urine. Twenty microliters of 0.4 g/mL sample extract was used. Plots of the ion trap CID transition of *m*/*z* 335 to 299 (top) and of *m*/*z* 435 to 375 (bottom) are shown.

The method was used on diagnostic samples. In one case, a goat was exposed to a plant that was identified as a *Rhododendron* species. Clinical signs included vomiting, depression, and brachycardia. The rumen content was positive for grayanotoxins by two-dimensional TLC (*15*). Urine and feces samples were not available. Subsequent LC-MS analysis of rumen content confirmed the presence of GTX I at 2.4 μg/g but found no GTX III (Figure 8). While *R. maximum* has been reported to contain both GTX I and GTX III at >50 μg/g (*16*), LC-MS analysis of the one sample of *R. maximum* found 74 μg/g GTX I, 1 μg/g GTX II, and 4 μg/g GTX III. One sample of *R. percii* contained no GTX I, 4 μg/g GTX II, and 140 μg/g GTX III. Thus, the amount of each grayanotoxin in leaf material appears to be variable and species dependent. This method was also used to identify grayanotoxins in feces and urine from goats exposed to *Rhododendron* (*17*).

LC-MS/MS can provide a rapid quantitative analysis of biological samples for grayanotoxins following a minimal SPE cleanup. This method is an improvement over the existing TLC method by providing quantitative results with mass spectral confirmation. The method can successfully be used to test rumen content, feces, and urine to demonstrate dietary exposure to grayanotoxin-containing plants. The procedure is especially suited to veterinary diagnostic laboratory situations for

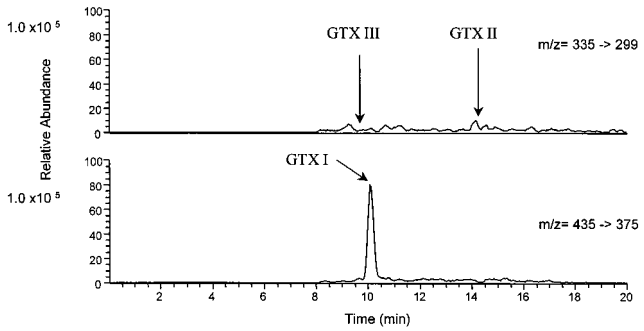

Figure 8. LC-MS/MS chromatogram of extract of caprine rumen sample positive for GTX I at 2.4 μg/g. Twenty microliters of 0.4 g/mL sample extract was used. Plots of the ion trap CID transition of *m*/*z* 335 to 299 (top) and of *m*/*z* 435 to 375 (bottom) are shown.

which rapid diagnosis of exposure to cardiotoxic plants is necessary. Future work will include expanding the method to include additional cardiotoxic compounds.

ACKNOWLEDGMENT

We thank W. H. Tallent, U.S. Department of Agriculture, Beltsville, MD, for providing the grayanotoxin standards.

LITERATURE CITED

(1) Cheeke, P. R. *Natural Toxicants in Feeds, Forages, and Poisonous Plants*; Interstate Publishers: Danville, IL, 1998; pp 390−393.

(2) Kingsbury, J. M. *Poisonous Plants of the United States and Canada*; Prentice-Hall: Englewood Cliffs, NJ, 1964; pp 251−260, 264−266, and 300−303.

(3) Hikino, H.; Ohta, T.; Ogura, M.; Ohizumi, Y.; Konno, C.; Takemoto, T. Structure−activity relationship of Ericaceous toxins on acute toxicity in mice. *Toxicol. Appl. Pharmacol.* **1976**, *35*, 303−310.

(4) Burke, J. W.; Doskotch, R. High field ^{1}H- and ^{13}C-NMR assignments of grayanotoxins I, IV and XIV isolated from *Kalmia augustifolia*. *J. Nat. Prod.* **1990**, *53*, 131−137.

(5) Smith C. M. Japanese Pieris poisoning in the goat. *J. Am. Vet. Med. Assoc.* **1978**, *173*, 78−79.

(6) Onat, F. Y.; Yegen, B. C., Lawrence, R.; Oktay, A.; Oktay, S. Mad honey poisoning in man and rat. *Rev. Environ. Health* **1991**, *9*, 3−9.

(7) Hough, I. Rhododendron poisoning in a western grey kangaroo. *Aust. Vet. J.* **1997**, *75* (March), 174−175.

(8) Sayama, I.; Narahashi, T. Modulation of sodium channels of squid nerve membranes by grayanotoxin I. *J. Pharmacol. Exp. Ther.* **1981**, *219*, 614−624.

(9) Terai, T. Determination of grayanotoxins I, II and III by thin-layer chromatography. *Nippon Nogeikagaku Kaishi* **1984**, *58*, 1117−1122.

(10) Kinghorn, A. D.; Jawad, F. H.; Doorenbos, N. J. Thin-layer chromatographic and spectroscopic determination of some diterpenes of the grayanotoxin type. *J. Chromatogr.* **1978**, *147*, 229−308.

(11) Sutlupinar, N.; Mat, A.; Satganoglu, Y. Poisoning by toxic honey in Turkey. *Arch. Toxicol.* **1993**, *67*, 148−150.

(12) Kerkvliet, J. D. Analysis of a toxic rhododendron honey. *J. Apic. Res.* **1981**, *20*, 249−253.

(13) Terai, T.; Katai, M.; Meguri, H. Quantitative analysis of grayanotoxins. I. Spectrometric determination of grayanotoxin I, II, and III. *Yakagku Zasshi* **1983**, *103*, 686−689.

(14) Terai, T.; Uda, T.; Katakawa, J.; Tetsumi, T. Assay of grayanotoxins by gas chromatography. *Nippon Nogeikagaku Kaishi* **1994**, *68*, 979−981.

(15) Holstege, D. M.; Francis, T.; Puschner, B.; Booth, M. C.; Galey, F. D. Multiresidue screen for cardiotoxins by two-dimensional thin-layer chromatography. *J. Agric. Food Chem.* **2000**, *48*, 60−64.

(16) Jawad, F. H.; Kinghorn, A. D.; Doorenbos, N. J.; Billets, S. Characterization and mass spectral analysis of some grayanotoxin derivatives. *Biomed. Mass Spectrom.* **1977**, *4*, 331−336.

(17) Puschner, B.; Holstege, D. M.; Lamberski, N.; Le, T. Grayanotoxin poisoning in 3 Nubian goats. *J. Am. Vet. Med. Assoc.* **2001**, in press.

Received for review June 21, 2000. Revised manuscript received December 5, 2000. Accepted December 5, 2000.

JF000750S