JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Determination of Oleandrin in Tissues and Biological Fluids by Liquid Chromatography−Electrospray Tandem Mass Spectrometry

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A rapid LC-MS/MS method, using a triple-quadrupole/linear ion trap mass spectrometer, was developed for the quantitative determination of oleandrin in serum, urine, and tissue samples. Oleandrin, the major cardiac glycoside of oleander (*Nerium oleander* L.), was extracted from serum and urine samples with methylene chloride and from tissues with acetonitrile. The tissue extracts were cleaned up using Florisil solid-phase extraction columns. Six replicate fortifications of serum and urine at 0.001 μg/g (1 ppb) oleandrin gave average recoveries of 97% with 5% CV (relative standard deviation) and 107% with 7% CV, respectively. Six replicate fortifications of liver at 0.005 μg/g (5 ppb) oleandrin gave average recoveries of 98% with 6% CV. This is the first report of a positive mass spectrometric identification and quantitation of oleandrin in tissue samples from oleander intoxication cases. The sensitivity and specificity of the LC-MS/MS analysis enables it to be the method of choice for toxicological investigations of oleander poisoning.

KEYWORDS: Oleander; mass spectrometry; LC-MS; 4000 Q TRAP; urine; serum; liver; heart

INTRODUCTION

Oleandrin, [16β-(acetyloxy)-3β-[(2,6-dideoxy-3-*O*-methyl-L-*arabino*-hexopyranosyl)oxy]-14-hydroxycard-20-(22)-enolide], $C_{32}H_{48}O_9$, MW 576.3 [CAS Registry No. 465-16-7], **Figure 1**, is the most prominent of several toxic cardiac glycosides found in all parts of the plant oleander, *Nerium oleander* L., Apocynaceae. Oleander poisoning cases in humans and livestock have been reported worldwide (*1*−*9*). Most poisonings in livestock result from accidental ingestion of oleander clippings, but oleander leaves may be baled with hay or chopped into silage and present a significant risk to animals (*1*, *2*). The oral, median toxic dose of ground oleander plant in horses and cattle was estimated to be 26 and 45 mg/kg of body weight, respectively (*2*). Clinical signs of oleander poisoning are well documented and include acute gastrointestinal (GI), neurological, and cardiovascular problems (*1*−*3*, *7*), followed by death in many cases.

Several methods for analysis of oleandrin have been developed. However, most analytical methods such as thin-layer chromatography (*1*, *10*, *11*), HPLC of the fluorescent derivative (*12*), or immunoassays for digoxin and digitoxin (*13*) are not sufficiently sensitive (method detection limit of 50 ppb in GI contents) or specific to detect and quantify oleandrin in biological fluids and tissues. In clinical cases of animals or humans displaying signs of oleander poisoning a rapid, highly sensitive, and specific method is needed to detect oleandrin at toxicologically significant, low parts per billion concentrations in a variety of biological matrices. This is especially important in forensic situations where the unequivocal identification of oleandrin is necessary. Biological fluids have been analyzed for oleandrin from human intoxication cases by liquid chromatography−mass spectrometry (LC-MS) techniques. These include single-quadrupole (*14*, *15*), ion trap (*16*), and tandem quadrupole time-of-flight mass spectrometers (*17*). The analysis of liver tissue for oleandrin from a human via LC−triple-quadrupole mass spectrometry has been reported with limited

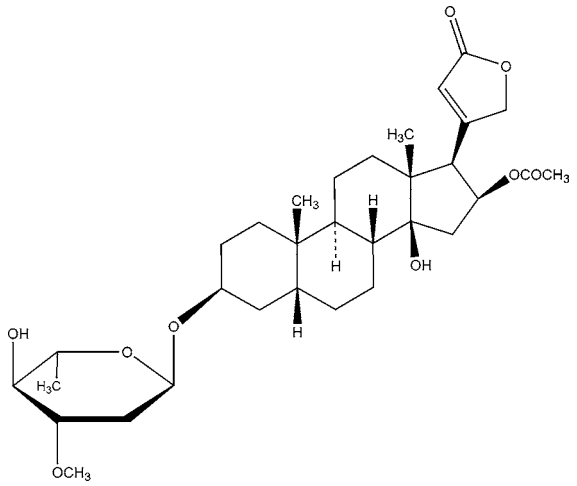

Figure 1. Oleandrin, a toxic cardiac glycoside found in oleander, *Nerium oleander* L., Apocynaceae.

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10.1021/jf050201s CCC: $30.25
Published on Web 04/29/2005

sensitivity (30 ppb detection limit) for oleandrin in fortified control human liver (*18*).

This investigation was undertaken to develop a sensitive and highly specific analytical method for the extraction, purification, and quantitative determination of oleandrin in tissues and biological fluids using a new LC−tandem triple quadrupole/linear ion trap mass spectrometer. The performance of the hybrid linear ion trap system was evaluated with regard to its ability to generate accurate and precise qualitative and quantitative data. The method was applied for diagnosing oleander poisoning cases in cattle.

MATERIALS AND METHODS

Reagents. Water, methanol, formic acid, and acetonitrile were of HPLC grade (Fisher Scientific); methylene chloride, hexane, and ethyl acetate were of Optima grade (Fisher Scientific). All HPLC running solvents were filtered through 0.45 μm nylon filters (Gelman Sciences, Ann Arbor, MI).

Preparation of Standard Solutions. Oleandrin standard was purchased from Sigma Chemical Co. (St. Louis, MO) (> 95% purity). A stock solution of 1000 μg/mL was made in methanol. Subsequent dilutions of the standard were made daily in methanol from the stock solution. Five-point calibration curves in serum, urine, or liver extracts were prepared at 0.0025, 0.005, 0.025, 0.05, and 0.1 μg/mL by adding aliquots of oleandrin standard to dry, negative control serum, urine, or liver extracts, prepared as described below.

Sample Preparation. *(a) Serum, Urine.* One gram of sample was weighed into a 15 mL, screw-cap, disposable tube, 10 mL of methylene chloride was added, and the sample was vigorously mixed by hand for 2 min. The mixture was centrifuged at 2000 rpm (260*g*) for 5 min using an IEC Centra-7 centrifuge (International Equipment Co., Needham, MA). An aliquot (9 mL) of the methylene chloride extract was evaporated to dryness, using an N-Evap nitrogen evaporator (Analytical Evaporator, Organomation Associates Inc., Berlin, MA) set at 40 °C. The extract was redissolved in 200 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.

(b) Liver, Heart Muscle. Ten grams of sample was weighed into a 250 mL French, squared homogenization vessel (Fisher Scientific). The sample was homogenized with 100 mL of acetonitrile for 1 min at 9500 rpm using an Ultra-Turrax T-25 tissue homogenizer (IKA-Labortechnik/Tekmar Co., Cincinnati, OH) and centrifuged at 500 rpm (65*g*) for 5 min as above. An aliquot (25 mL) of the clear extract was evaporated to dryness as above. The dry extract was redissolved by vortexing in 1 mL of hexane and quantitatively transferred onto a 1000 mg, 6 cm^3, Florisil SPE cartridge (Baker, Phillipsburg, NJ), prewashed with 10 mL of hexane. The column was washed with 10 mL of 5% ethyl acetate in hexane (v/v); this wash was discarded. Oleandrin was eluted from the column with 15 mL of ethyl acetate into a glass, disposable test tube. The eluate (extract) was evaporated to dryness as above, redissolved in 250 μL of methanol, and filtered into an autosampler vial. All control and fortified samples were prepared in the same manner.

LC-MS/MS Analysis. An Agilent model 1100 (binary) high-performance liquid chromatograph coupled with a hybrid triple-quadrupole/linear ion trap mass spectrometer, model 4000 Q TRAP (Applied Biosystems/MDS SCIEX, Concord, Canada) was used in all analyses. The analytical column was a 150 mm × 4.6 mm × 5 μ Luna C18(2) (Phenomenex Inc., Torrance, CA), with a C18 guard column cartridge. The injection volume was 20 μL. The mobile phase consisted of (A) 0.1% formic acid in water (v/v) and (B) 0.1% formic acid in methanol (v/v) at a flow rate of 500 μL/min under a linear gradient of 75% B to 95% B over 12 min. The retention time for oleandrin was 7.2 min. MS data were acquired in the positive ion electrospray ionization (ESI) mode, using the following TurboIonSpray source conditions: temperature, 400 °C; curtain gas, 30 (arbitrary units); GS1 and GS2, 50; CAD gas pressure, low; ion spray voltage, 5500. The precursor ion of *m*/*z* 577 was selected in Q1 and accelarated into the pressurized LINAC collision cell (Q2): collision energy (CE), 20; declustering potential (DP), 50; collision energy spread (CES), 5.0, followed by trapping and mass analysis in the Q3 linear ion trap, using the enhanced product ion (EPI) scan function; dynamic fill time, on; Q3 entry barrier, 8 V, acquiring the product ion spectrum in the range of *m*/*z* 100−440. These parameters were optimized while 1 μg/mL oleandrin standard at 10 μL/min was infused into the mobile phase as above. Twenty 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 five-point calibration curve using external standards in matching matrix and nonweighted linear regression using the Analyst version 1.4 software.

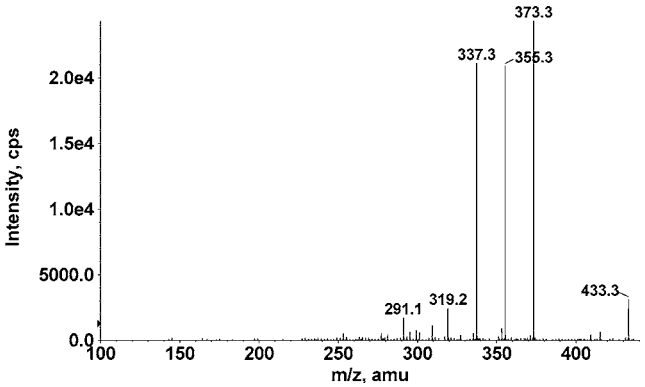

Figure 2. Positive ion ESI-MS/MS product ion spectrum of oleandrin in pure analytical standard prepared in methanol. The MS/MS spectrum was obtained by fragmenting the protonated molecular ion of *m*/*z* 577. Oleandrin eluted from the HPLC column at 7.22 min.

Method Validation. The method was validated by analyzing control bovine serum, urine, or liver samples ($n = 6$ each) fortified with oleandrin at 0.001 μg/g (serum and urine) and at 0.005 μg/g (liver). The fortifications were prepared by adding 20 μL of 0.05 μg/mL oleandrin standard to 1 g of negative, control bovine urine and serum samples and by adding 50 μL of 1 μg/mL oleandrin standard to 10 g of negative, control bovine liver samples, respectively, and analyzing them using the method described above. The method was also tested by routine analyses of samples from veterinary diagnostic cases.

RESULTS AND DISCUSSION

The combination of the highly selective triple-quadrupole mass spectrometer with the high sensitivity linear ion trap on the same instrument platform provided reliable quantitation and confirmation of the presence of oleandrin in complex biological matrices at toxicologically significant, low parts per billion levels. Protonation was the main ionization process observed in the positive ESI mass spectrum of oleandrin obtained during the infusion of the 1 μg/mL oleandrin standard into the mobile phase, with $[M + H]^+$ (*m*/*z* 577) as the base peak.

Figure 2 shows a typical LC-MS/MS product ion spectrum of oleandrin standard in methanol. The MS/MS spectrum was obtained by fragmenting the protonated molecular ion of *m*/*z* 577. The most prominent product ions at *m*/*z* 433 (oleandrigenin, $C_{25}H_{37}O_6$, the aglycon resulting from a loss of monosaccharide residue), 373, 355, and *m*/*z* 337 were observed under the EPI scan conditions described. This was similar to the product ion spectrum of oleandrin previously described (*17*). The sum of *m*/*z* 373, 355, and 337 ions was used for quantitation. Five picograms of oleandrin standard in methanol on column gave an S/N of 22. Quantitation of oleandrin in urine and serum using electrospray ionization was affected by ion suppression. The most significant suppression (12%) was observed in urine matrix. Therefore, it was essential to perform quantitation using standards in matrices that matched those of the samples. The

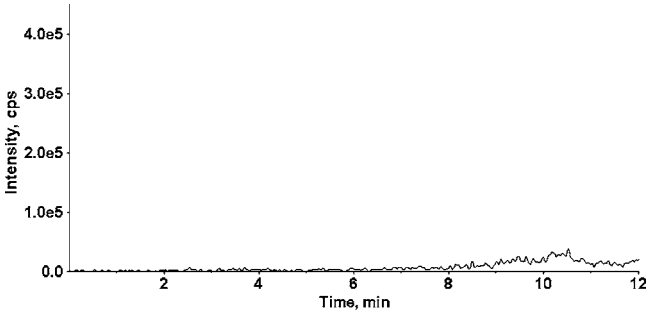

Figure 3. Typical LC-MS/MS chromatogram (sum of *m*/*z* 373, 355, and 337) of a negative control bovine liver.

standard curves in matrix followed linear regression with r^2 values of 0.9982 (serum), 0.9994 (urine), and 0.9990 (liver). This was similar to the standard curve in methanol, which followed linear regression with an r^2 value of 0.9998. These results demonstrate that the performance of the hybrid triple-quadrupole/linear ion trap system was sufficient to provide accurate and precise quantitative analysis of oleandrin in biological matrices.

Six replicate fortifications of serum and urine at 0.001 μg/g oleandrin gave average recoveries of 97% with 5% CV (relative standard deviation) and 107% with 7% CV, respectively. Six replicate fortifications of liver at 0.005 μg/g oleandrin gave average recoveries of 98% with 6% CV.

Confirmation (qualitative identification) was based on the ion ratios of the MS/MS product ions in samples matching those in the standards and spikes. The hybrid triple-quadrupole/linear ion trap system provided consistent MS/MS ion ratios, allowing for a high level of confidence in oleandrin identification. Peak height ratios for the product ions at *m*/*z* 337 and 355 were each calculated against the peak height of the base peak at *m*/*z* 373. The average peak height ratio for *m*/*z* 337/373 was 0.83 (range = 0.75−0.89, CV = 5.5%), and the average peak height ratio for *m*/*z* 355/373 was 0.85 (range = 0.78−0.90, CV = 4.8%) from analyses of $n = 10$ standards at concentrations of 0.005−0.1 μg/mL prepared in methanol. This was compared to ion ratios from analyses of standards of the same concentrations prepared in liver extract ($n = 15$), where the average peak height ratio for *m*/*z* 337/373 was 0.86 (range = 0.81−0.91, CV = 3.2%) and that for *m*/*z* 355/373 was 0.86 (range = 0.76−0.91, CV = 4.8%). Therefore, no significant differences in ion ratios caused by liver matrix were observed. Ion ratios for oleandrin in diagnostic samples were within 10% of the means of the ion ratios from the associated analytical standards. These data indicate that the hybrid triple-quadrupole/linear ion trap system can provide qualitative data that exceed strict method performance criteria such as those specified in the European Union guidelines for analysis of veterinary drug residue analysis, with allowable CV for relative ion intensities of 20% (*19*).

The use of tandem triple-quadrupole/linear ion trap mass spectrometry produced clean chromatograms and spectra of oleandrin with minimal background contribution from urine or serum matrix. A challenging aspect was the analysis of tissue samples at low parts per billion concentrations. Tissue samples are often the only available specimens in toxicological investigations. The Florisil SPE column removed matrix interferences and offered an efficient sample cleanup of liver and heart tissues. LC-MS/MS ion chromatograms and spectra of oleandrin in a control negative bovine liver and control bovine liver fortified with oleandrin at the 0.001 μg/g, (1 ppb) are shown in **Figures 3** and **4** to demonstrate instrument sensitivity and cleanup efficiency.

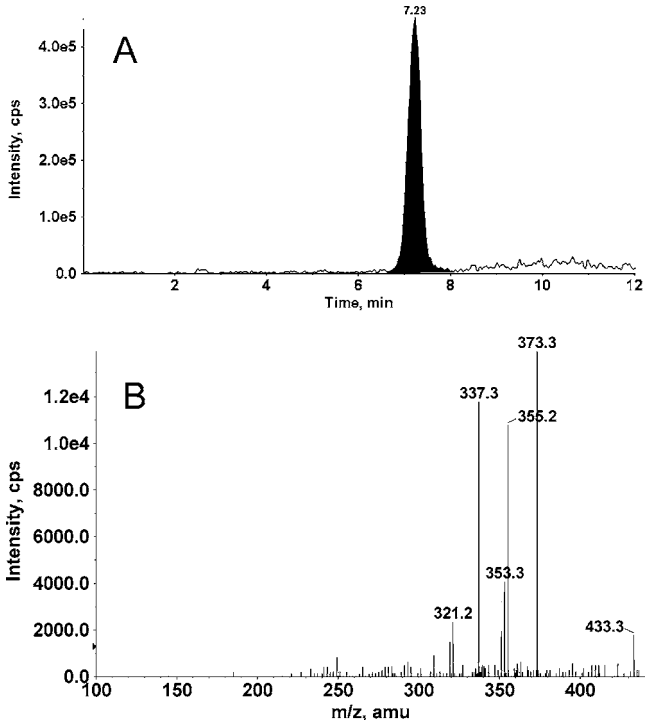

Figure 4. (**A**) LC-MS/MS chromatogram (sum of *m*/*z* 373, 355, and 337) of control bovine liver fortified with oleandrin at 0.001 μg/g (1 ppb), S/N = 85. (**B**) Positive ESI-MS/MS spectrum of the peak at retention time of 7.23 min.

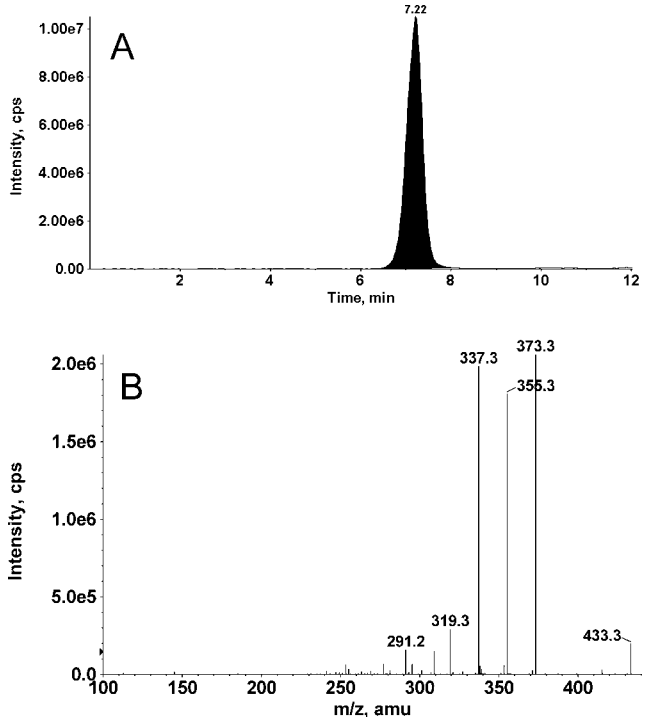

Figure 5. (**A**) LC-MS/MS chromatogram (sum of *m*/*z* 373, 355, and 337) and (**B**) positive ESI-MS/MS spectrum of oleandrin in liver sample from a cow accidentally exposed to oleander. The concentration of oleandrin in the liver was 0.039 μg/g (39 ppb). The retention time was 7.22 min.

The described method was applied to the analysis of serum, urine, and tissue samples from veterinary diagnostic samples. **Figure 5A** shows a chromatogram of a bovine liver extract that tested positive for oleandrin at 0.039 μg/g (39 ppb). The liver was analyzed in duplicate with a CV of 2%. Oleandrin was clearly identified in the liver sample by comparing the MS/MS

product ion spectra to that of an analytical standard (**Figure 5B**). The peak height ratio for *m*/*z* 337/373 was 0.91 and that for *m*/*z* 355/373 was 0.86. Oleandrin was also present in the heart muscle from the same animal at a concentration of 0.021 μg/g (21 ppb). The retention time and spectrum of oleandrin in heart muscle were identical to these in the liver. The liver and heart above were reanalyzed for oleandrin after storage of these samples at −20 °C for 5 months. The results were identical (CV = 2%), proving no loss of oleandrin during sample storage. A second cow from the same herd contained 0.053 μg/g (53 ppb) of oleandrin in the liver and 0.004 μg/g (4 ppb) of oleandrin in urine. The urine sample was analyzed in triplicate with a CV of 4%, with the average ion ratio for *m*/*z* 337/373 of 0.85 and that for *m*/*z* 355/373 of 0.90. In both cows, oleander poisoning was not suspected until a post-mortem examination. The detection of oleandrin in tissues and urine confirmed a diagnosis of oleander toxicosis and led to an in-depth investigation as to the source of the oleander.

This method is a significant improvement over the existing methodology in that it provides a rapid and unequivocal determination of oleandrin in biological fluids and it is validated at very low, parts per billion concentrations. Additionally, it offers the first report of a positive mass spectrometric identification and quantitation of oleandrin in liver and heart samples from an oleander intoxication case, along with firm quality control measures in these difficult matrices. Little is known about the kinetics of oleandrin in animals, but the applied methodology demonstrated the distribution of oleandrin to liver and heart at significant concentrations. The newly developed method can be applied to establish kinetic parameters of oleandrin. Batches of 50 urine or serum or 10 tissue samples can be easily extracted and analyzed by one analyst in a day. The sensitivity and specificity of the 4000 Q TRAP mass spectrometer makes the described method a powerful tool for forensic−toxicological investigations in human and veterinary medicine. Serum and urine from animals or humans with suspected oleander poisoning can rapidly be analyzed by using this technique, providing key information to the clinician and increasing the chance for survival. In post-mortem cases, the developed method allows for the confirmation of oleandrin in liver and heart. This methodology would also be valuable for the confirmation of oleander intoxication in human clinical cases in which immunoassays for digoxin or digitoxin are used as screening tools, especially if such cases involve legal or public health investigations.

ACKNOWLEDGMENT

We thank Drs. Leslie Woods and Asheesh Tiwary from the California Animal Health and Food Safety Laboratory System for providing diagnostic material and many helpful suggestions.

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Received for review January 27, 2005. Revised manuscript received March 21, 2005. Accepted March 23, 2005.

JF050201S