Determination of Oleander Glycosides in Biological Matrices by High-Performance Liquid Chromatography

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A high-performance liquid chromatography (HPLC)−fluorescence method was developed for quantitative determination of oleandrin in gastrointestinal contents (stomach, rumen, colon, and cecum contents). Oleandrin was extracted with methylene chloride. The extract was cleaned up on a charcoal solid phase extraction (SPE) column, derivatized with 1-naphthoyl chloride, and quantified by HPLC with a fluorescence detector. The limit of detection was 0.05 ppm. Six replicate fortifications of stomach contents matrix at 0.10 ppm oleandrin gave an average recovery of 85%, with 4.6% CV (relative standard deviation). The diagnostic utility of the method was tested by analyzing samples submitted to the veterinary toxicology laboratory.

Keywords: *Oleandrin; HPLC determination; oleander glycosides*

INTRODUCTION

Fatal oleander *(Nerium oleander* L.) poisoning has been reported in many areas of the world (Alfonso et al., 1994; Rezakhani and Maham, 1992; Pahwa and Chatterjee, 1990). Accidental poisoning in humans has occurred from chewing the flowers and eating meat cooked over oleander branches or food stirred with oleander stems (Szabuniewicz et al., 1971). Ingestion of plant clippings is the most frequent source of oleander poisoning in livestock (Galey et al., 1996; Alfonso et al., 1994). The toxicity of oleander plant is attributed to cardiac glycosides, mainly oleandrin (Figure 1), neriin, and adynerin (Wagner et al., 1984; Seiber et al., 1983; Majak, 1992; Schwartz et al., 1974). The toxic action, metabolism, chemistry, and structure−activity relationships of cardiac glycosides were reviewed by Greeff (1981) and Bodem and Dengler (1978).

Clinical signs of oleander poisoning in livestock are nonspecific. Currently, diagnosis of oleander poisoning relies on identification of leaves or other parts of the plant in the gastrointestinal (GI) contents or finding the plant clippings in the feed. Recently, a two-dimensional thin-layer chromatography (TLC) screen of GI contents for oleandrin was developed in this laboratory to aid in the diagnosis of oleander poisoning in livestock (Galey et al., 1996). A reliable analytical method for the quantitation and confirmation of oleandrin at nanogram levels is needed. The majority of analytical methods are devoted to cardiac glycosides from the digitalis (foxglove) plant (see review by Vatticaden and Chandrasekaran, 1990). Only a few methods for analysis of oleander glycosides have been developed (Blum and Rieders, 1987; Karawya et al., 1970). These methods lack the sensitivity or specificity necessary to detect oleandrin in GI contents at toxicologically significant levels. The liquid chromatography−mass spectroscopy (LC/MS) technique (Rule et al., 1993) is very specific and sensitive but is not readily available in diagnostic laboratories. A high-performance liquid chromatography (HPLC) method for quantitation of oleandrin in samples detected by the two-dimensional TLC technique is described in this paper. This method is designed for relatively quick turnaround of large sample sets and has been tested on samples presented to the veterinary diagnostic laboratory.

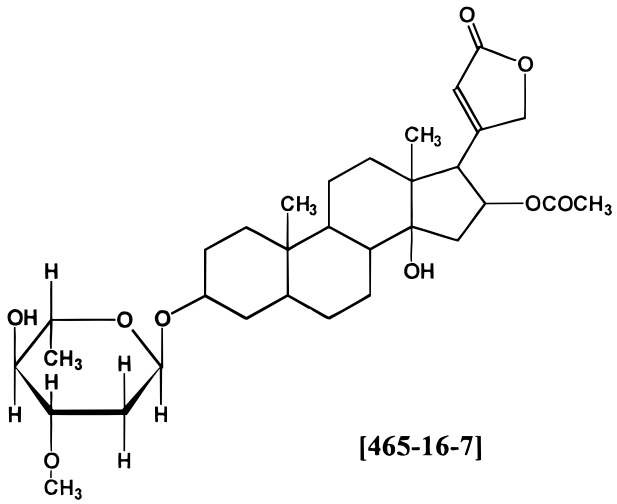

Figure 1. Structure and CAS Registry number of oleandrin.

EXPERIMENTAL PROCEDURES

Apparatus and Equipment. A Hewlett-Packard model 1090 HPLC with a Waters model 474 fluorescence detector (excitation, 220 nm; emission, 345 nm) was used in all HPLC analyses. The analytical column was a Spherisorb C18, (15 cm × 4.6 mm × 5 μM particle size; Phase Sep., Norwalk, CT), with HP guard column cartridge (MOS Hypersil, 2 cm × 4 mm × 5 μM). The oven temperature was 50 °C. The premixed mobile phase used was 35% water in acetonitrile at a flow rate of 1 mL/min. The injection volume was 20 μL. Under these conditions, the retention time for the oleandrin derivative was 18.2 min. Quantification was done by comparison with external standard peak areas generated by the PE Nelson Turbochrom Version 3 data acquisition system (Cupertino, CA).

Reagents. 1-Naphthoyl chloride, 97% pure, was obtained from Aldrich Chemical Company (Milwaukee, WI). 4-Dimethylaminopyridine (4-DMAP) was from Sigma Chemical Company (St. Louis, MO). All solvents were Optima grade (Fisher Scientific). The HPLC solvents were filtered through 0.45-μM nylon filters (Gelman Sciences, Ann Arbor, MI). Sodium sulfate was ACS reagent grade (Fisher Scientific).

Preparation of Standard Solutions. Oleandrin standard was obtained from Sigma Chemical Co. (St. Louis, MO). A standard solution of 1000 μg/mL in methanol was stable for 6 months when stored at 5 °C. Subsequent dilutions of the standard in methanol were made daily from the stock solution.

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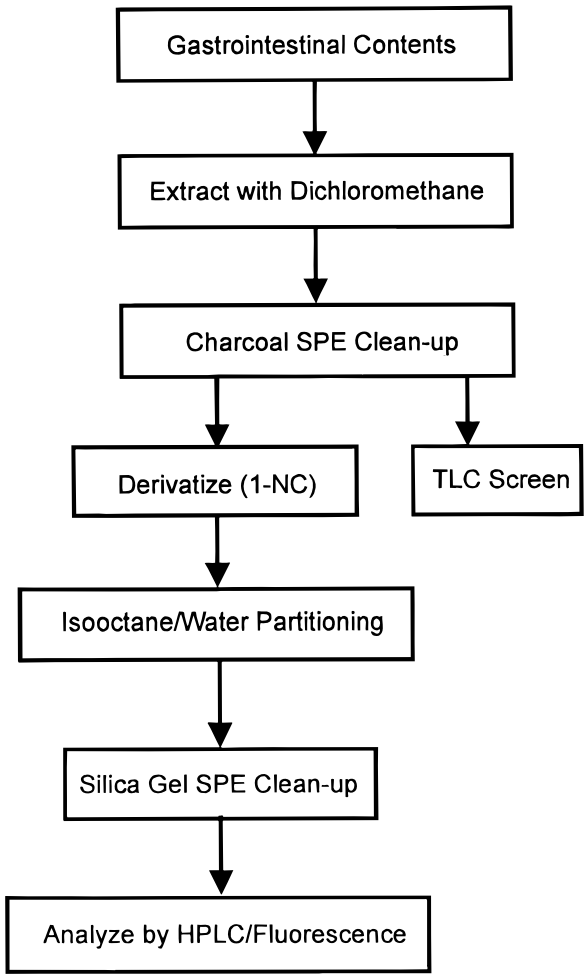

Figure 2. Flow diagram of the overall analytical procedure.

Procedure. A flow diagram of the overall method is presented in Figure 2. The details are as follows.

(a) Extraction of Oleandrin from GI Contents (Stomach, Rumen, Colon, and Cecum Contents). Ten grams of sample was weighed into a 250 mL French, squared homogenization vessel (Fisher Scientific). Methylene chloride (100 mL) and sodium sulfate (50 g) were added to each of the samples. Samples were homogenized for 1 min at 9500 rpm with a tissue homogenizer (model Ultra-Turrax T-25, IKA-Labortechnik/Tekmar Company, Cincinnati, OH). The extracts were centrifuged at 500 rpm (65*g*) for 5 min with an IEC Centra-7^R centrifuge (International Equipment Company). Aliquots (25 mL) were evaporated just to dryness with a nitrogen evaporator (N-Evap, Analytical Evaporator, Organomation Associates Inc., Berlin, MA) set at 40 °C. The dry extracts were redissolved in 1 mL of 15% methanol in methylene chloride (v/v).

(b) Charcoal Solid-Phase Extraction (SPE) Cleanup. Charcoal SPE columns (MycoSep; Romer Laboratories Inc., Union, MO) were washed before use with 10 mL of methylene chloride. Samples were loaded onto the column in 15% methanol in methylene chloride (v/v). Oleandrin was eluted from the column with 20 mL of methylene chloride. The cleaned-up extract was evaporated to dryness as described above and redissolved in 50 μL of methanol.

(c) TLC Analysis. Twenty microliters of sample (1 g equivalent) was spotted on the silica gel TLC plate and analyzed as described by Galey et al. (1996). Briefly, the TLC plate was developed with a mixture of solvents, sprayed with 20% aluminum chloride in water:ethanol (1:1), and heated for 5 min at 110 °C. Oleandrin appeared as a blue spot under long wavelength ultraviolet (UV) light (366 nm). The remaining extracts from samples that were positive by TLC (1.5 g equivalent) were evaporated to dryness and analyzed by HPLC as described in section *g*.

(d) Derivatization for Fluorescence Detection. 1-Naphthoyl chloride solution in acetonitrile (1:20, v/v) was prepared daily and kept in a dark glass container. Four hundred microliters of the 1-naphthoyl chloride solution and 60 mg of 4-DMAP were added to the dry extracts. Samples were mixed by vortex for 10 s to ensure complete dissolution of the residue. The test tubes were tightly capped and placed in a 80 °C water bath for 45 min.

(e) Liquid/Liquid Cleanup. Derivatized samples were dissolved in 2 mL of distilled water. Isooctane (4 mL) was added. The samples were rotated on a rotary shaker (model Reax 2, Heidolph, Germany) for 10 min to extract the derivatives and centrifuged at 2300 rpm (1200*g*) for 5 min. Two milliliters of the isooctane layer were transferred into a clean test tube, evaporated to dryness, and made up in 1 mL of methylene chloride.

(f) Silica Gel SPE Cleanup. Derivatives were cleanedup further with silica gel SPE columns (500 mg, 6 cc; Waters Corporation, Milford, MA). Columns were washed before use with 10 mL of methylene chloride. Samples were loaded in 1 mL of methylene chloride, and the columns were washed with 9 mL of methylene chloride to remove interferences (this wash was discarded). Oleandrin was eluted from the columns with 6 mL of 10% methanol/methylene chloride (v/v). The eluates were evaporated to dryness, redissolved in 0.15 mL of mobile phase (35% water/acetonitrile, v/v) and filtered through a 0.45-μM HPLC filter (Millipore Corporation, Milford, MA) into Hewlett-Packard autosampler vials.

(g) HPLC−Fluorescence Determination of Oleandrin−1-Naphthoyl Chloride Derivative (OLE−1NC). Analytical standards of OLE−1NC at concentrations ranging from 0.01 to 0.75 μg/mL, which were derivatized along with the samples, were injected into the HPLC system as described above. Each set of samples analyzed also contained a reagent blank, a control and a fortified sample. Quantitation was done with external calibration based on injections of 0.2−15 ng of OLE−1NC standards.

Method Validation. Rumen contents from 10 animals not exposed to oleander (controls) were collected at necropsy, stored at −20 °C for 2 months, and analyzed to examine the background level of chromatographic interferences. The method was validated by analyzing control bovine rumen content samples (n = 6) fortified with oleandrin at 0.1 ppm. The method was also tested by analyzing samples from diagnostic cases.

RESULTS AND DISCUSSION

Diagnosis of oleander poisoning in livestock at the California Veterinary Diagnostic Laboratory System has been greatly improved since the development of chemical tests for oleander glycosides. The HPLC method described herein provided additional evidence of the presence of oleandrin in veterinary diagnostic samples that tested positive by the two-dimensional TLC screen. Using this new method, oleandrin was quantified in equine stomach content (0.44 ppm) from an animal that injested oleander clippings. Proventricular contents from a rhea that died suddenly after injesting dried oleander leaves contained 7.8 ppm of oleandrin. Oleandrin was quantified in rumen and cecal contents from a llama (0.2 and 0.1 ppm, respectively)that died suddenly. Diagnostic evidence of oleander poisoning in case material included clinical signs, case histories, pathological findings, and the results of the TLC screen (Galey at al., 1996). Fresh leaves of an oleander plant (*N. oleander* L.) collected in Davis, CA, contained 2200 ppm of oleandrin (n = 3, CV = 3.3%). The average recovery of oleandrin from bovine rumen fortified at 0.1 ppm was 85% (n = 6), with 4.5% RSD.

Silica gel SPE column cleanup was necessary to remove interferences in the OLE−1NC region of the GI contents. Typical chromatograms of 1NC derivatives

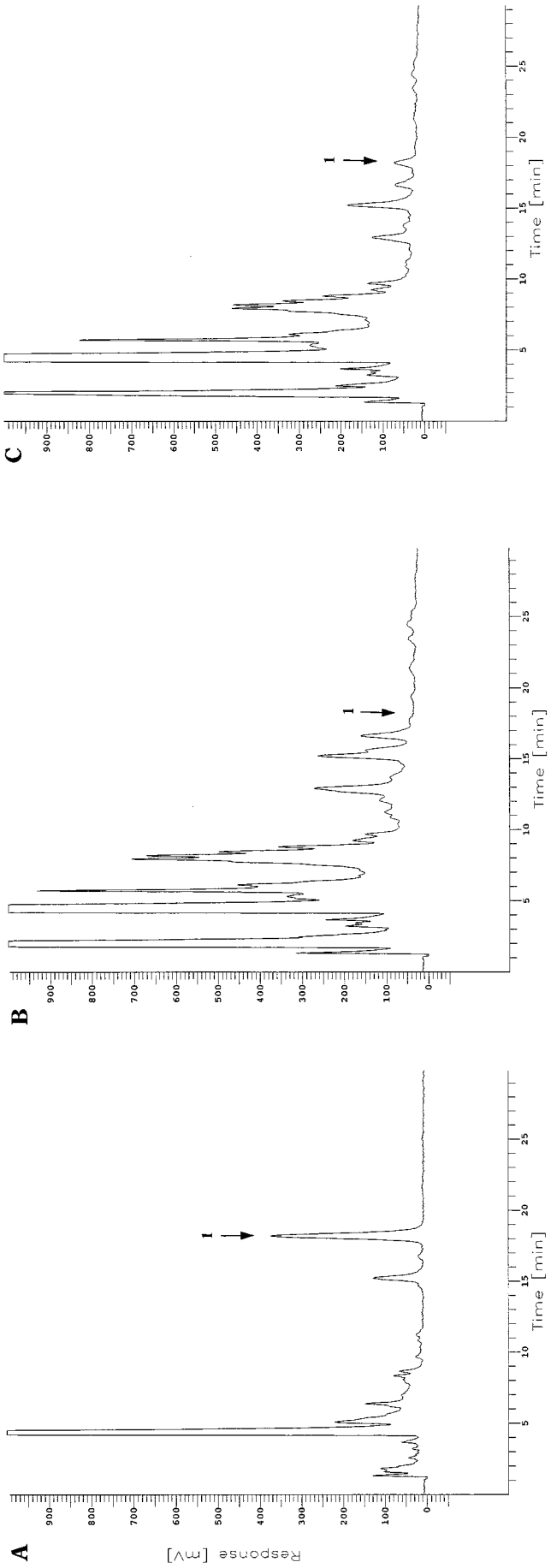

Figure 3. HPLC chromtograms of the 1-naphthoyl chloride derivative of oleandrin in (A) oleandrin standard, 2.5 ng; (B) control rumen, 50 mg matrix injected; (C) rumen spike at 0.1 ppm; (D) rumen from a llama exposed to oleander (0.2 ppm found); (E) cecum content from a llama exposed to oleander (0.1 ppm).

of oleandrin are shown in Figure 3A−E. A comparison of the chromatograms clearly shows the presence of oleandrin (1) in the rumen and cecum content of the llama just described. This result was consistent with the results of the TLC screen. Detecting oleandrin in cecal content samples by TLC/HPLC greatly enhanced the diagnosis of oleander poisoning since identification of oleander leaves in this matrix is difficult.

A challenging aspect of this analysis was the sensitivity needed to detect oleander glycosides at toxicologically significant (ppb) levels. This level of sensitivity is necessary because of the high toxicity of the plant (LD_{50}, 0.3 mg/kg; 10−20 leaves are enough to kill a healthy horse). A GC/MS technique with several silylation reagents was investigated, but resulted in poor stability and lack of sensitivity of the derivatives. Derivatization of oleandrin with 1NC in acetonitrile gave the best sensitivity and reproducibility among all HPLC derivatization reagents and conditions examined in this study. With the Waters model 474 fluorescence detector, 0.5 ng of OLE−1NC gave a 10:1 signal-to-noise ratio. The standard curves were linear, with r^2 ranging from 0.9340 to 0.9991. OLE−1NC was stable for 48 h at room temperature. Injections of derivatized samples did not produce adverse effects on the HPLC system. After 156 injections of sample extracts, there was no sign of a general decrease in column or instrument performance. Retention times over the course of the study varied by no more than 1%, and the peak areas for standard injections varied by <5%. It was important to use the premixed mobile phase. Small changes in mobile phase composition resulted in considerable shift in retention times (up to 3 min). The drawback of the method is a relatively long sample preparation time. This problem is encountered in all methods for quantitation of cardiac glycosides in plants.

The sensitivity of the method enables it to be used as a diagnostic tool in oleander poisoning cases. The two comparative identifications used, two-dimensional TLC and HPLC, constitute two chemically different principles and thus provide at least the minimal proof of identification. The detection of oleandrin in GI content samples depends on the time of sampling. A study is in progress to determine the impact of time on concentrations of oleandrin in the bovine GI tract. The limits of detection in all sample types were sufficiently low to quantitate the oleandrin in samples that tested positive by the TLC screen.

CONCLUSIONS

The method described proved to be suitable for quantitative analysis of oleandrin, the main cardiac glycoside from oleander, in GI contents. This method was validated by spiking experiments and also by application to analysis of samples from diagnostic cases submitted to the veterinary toxicology laboratory. The sensitivity (MDL, 0.05 ppm) of the method enables it to be applied to the diagnosis of oleander poisoning in animals.

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