A high-performance liquid chromatographic procedure with a postcolumn fluorescence derivatization is developed for the analysis of oleandrin in bovine blood. Oleandrin is separated by an octadecylsilane-bonded column with a mobile phase containing dehydroascorbic acid. The effluent of the column is mixed with concentrated hydrochloric acid and passed through poly(tetrafluoroethylene) tubing maintained at 70°C. The resultant fluorophores are detected at 465 nm with excitation at 348 nm. Simple solid-phase extraction using Sep-Pak tC2 is effective for sample purification. We found the minimal detectable quantity of oleandrin in plasma to be 1.5 ng/mL at a signal-to-noise ratio of 3:1.

Introduction

Oleander (Nerium oleander L., N. indicum Mill.) is a popular plant widely distributed throughout the world's subtemperate, subtropical, and tropical zones. In Japan, its hardiness and attractive flowers make it common as a garden tree and a plant along the highways. All parts of this plant are toxic to human beings and animals, although it is said that leaves and twigs are more toxic than other parts (1). Oleander is a rapidly growing plant and consequently needs frequent pruning. Ingestion of the leaves and branches cut off after trimming are a main cause of poisoning in animals.

The plant contains several kinds of cardiac glycoside, mainly oleandrin (Figure 1). Cardiac glycosides cause poisoning by inhibiting Na+K+ ATPase activity. Oleander is highly toxic and only 50 mg dry powdered leaf per kilogram body weight is lethal to cattle (2).

Thin-layer chromatographic (TLC) detection of oleandrin from gastric contents or urine has been reported for the diagnosis of poisoning (3). However, TLC methods require laborious sample preparation. Furthermore, sensitivity is not enough for the detection of blood oleandrin.

The analysis of blood oleandrin by high-performance liquid chromatography (HPLC) with UV detection has also been reported for the diagnosis of poisoning (4). The detection of oleandrin by UV absorption is simple but sensitivity and specificity is low. Mass spectrometric detection of oleandrin after HPLC separation (LC–MS) is highly specific (5) but requires expensive instruments. Tor et al. (6) applied the precolumn fluorescent label of oleandrin by 1-naphthoyl chloride for the determination of oleandrin in gastrointestinal contents. However, the sensitivity of their method is lower than UV detection (4), and derivatization at lower relative humidity is required for the effective production of fluorophore (7).

Wells et al. (8) reported the spectrofluorometric analysis of cardiac steroids by using the fluorogenic reaction of steroids with strong acid. The reaction mechanism is not known exactly, but dehydration reaction at the steroid portion of the cardiac glycoside is probably responsible. Several workers applied this reaction for the postcolumn derivatization of digoxin and developed an HPLC assay for blood digoxin (9–11).

Abstract

A high-performance liquid chromatographic procedure with a postcolumn fluorescence derivatization is developed for the analysis of oleandrin in bovine blood. Oleandrin is separated by an octadecylsilane-bonded column with a mobile phase containing dehydroascorbic acid. The effluent of the column is mixed with concentrated hydrochloric acid and passed through poly(tetrafluoroethylene) tubing maintained at 70°C. The resultant fluorophores are detected at 465 nm with excitation at 348 nm. Simple solid-phase extraction using Sep-Pak tC2 is effective for sample purification. We found the minimal detectable quantity of oleandrin in plasma to be 1.5 ng/mL at a signal-to-noise ratio of 3:1.
In this report, we optimized this postcolumn derivatization of cardiac glycosides for the determination of oleandrin in blood. Simple solid-phase extraction by a Sep-Pak tC2 cartridge was also examined.

**Experimental**

**Apparatus**

Fluorescence spectra were obtained with an RF5300PC fluorescence spectrophotometer (Shimadzu, Kyoto, Japan).

The HPLC postcolumn setup is shown in Figure 2. The chromatographic analysis was performed using an LC-10A system (Shimadzu) consisting of an LC-10AD pump, RF-10A fluorescence detector, and CTO-10A column oven. Chromatographic separations were performed on a Shimadzu STR ODS II column (4 × 150 mm). Hydrochloric acid was delivered by an HPLC pump LC-10Ai with a chemically inert pump head. The postcolumn derivatization with hydrochloric acid was performed in poly(tetrafluoroethylene) (PTFE) tubing (0.25 mm × 20 m) coupled to the column outlet by a T-piece mixer unit. The reactor was heated in a CRB-6A chemical reaction box. The outlet of the reactor was connected to the fluorescence detector. The original flow-through cell retainer was replaced by chemically inert parts supplied by Shimadzu.

**Reagents**

Oleandrin was purchased from Sigma-Aldrich Japan (Tokyo, Japan). HPLC-grade acetonitrile, concentrated hydrochloric acid, and the other chemicals were purchased from Nakarai Tesque (Kyoto, Japan). A Sep-Pak Light tC2 solid-phase extraction cartridge was supplied by Waters (Milford, MA).

A dehydroascorbic acid solution was prepared by adding hydrogen peroxide at the concentration of 4.3 mM to a 0.2% ascorbic acid solution. The mixture of ascorbic acid and hydrogen peroxide was stirred for 2 h to form dehydroascorbic acid. The prepared dehydroascorbic acid solution was effective for one week in an amber bottle at room temperature.

**Methods**

A 10-µg amount of oleandrin was dissolved in 3 mL of acetonitrile–dehydroascorbic acid (6:4). This solution was mixed with 3 mL of concentrated hydrochloric acid. After reaction at room temperature, emission and excitation spectra were recorded by using a fluorescence spectrophotometer.

Oleandrin in blood samples was purified by solid-phase extraction. A Sep-Pak Light tC2 column was preconditioned by 2 mL of methanol followed by 2 mL of water. A 0.5-mL volume of plasma was put on the column. After being washed with 2 mL of water and 2 mL of water–acetonitrile (8:2), oleandrin was eluted with 2 mL of water–acetonitrile (6:4). After drying under reduced pressure, samples were dissolved with 50 µL of methanol, and a 10-µL portion was injected to HPLC.

The eluent for the HPLC separation of oleandrin was acetonitrile–dehydroascorbic acid (6:4). The flow rate of the eluent was 0.4 mL/min. The temperature of the separation column was maintained at 40°C by a column oven. The flow rate of the derivatization medium (concentrated hydrochloric acid) was 0.4 mL/min. The fluorogenic reactor coil was kept at 70°C in a chemical reaction box.

The pump and tubing for hydrochloric acid delivery were rinsed with water at the end of every day's operation. The glass reservoir for concentrated hydrochloric acid was kept in a plastic container to avoid the diffusion of acid vapor.
Results and Discussion

Fluorescence spectra
The fluorescence spectra of an oleandrin derivative after batch reaction in acidic solution were obtained to get suitable wavelength settings for the chromatographic determination of oleandrin. As shown in Figure 3, fluorescence (excitation = 348 nm, emission = 465 nm) was induced at room temperature. The intensity of fluorescence was increased by the passage of time up to 90 min (Figure 4). From the results obtained, the excitation and emission wavelength of the HPLC fluorescence detector was set as 348 nm and 465 nm, respectively.

Optimization of postcolumn reaction
In order to accelerate the fluorogenic reaction observed at room temperature, the postcolumn reactor (PTFE tubing, 0.25 mm × 20 m) was heated in an oven. The flow rate of the reaction mixture (a mixture of column eluent and hydrochloric acid) and the reactor length both influenced the time available for derivatization reaction. The reaction temperature was another parameter for the derivatization reaction. In this study, we fixed the flow rate and the length of reaction coil to examine the optimal reaction temperature. As shown in Figure 5, maximum sensitivity was obtained by heating at 70°C. When the reactor was heated at higher than 70°C, fluorescence intensity was decreased. This suggested the decomposition of fluorophore by overheating. In order to achieve maximum sensitivity of detection, the concentrations of dehydroascorbic acid in the aqueous portion of the
mobility phase were altered while maintaining the quantity of hydrochloric acid constant. An optimal concentration of dehydroascorbic acid in the aqueous portion of the mobile phase was 0.2% (Figure 6).

**Preparation of blood samples**

Namera et al. (4) used an Extrelut column (Merck, Darmstadt, Germany) for the sample preparation, but this purification method was not effective for the present HPLC. We applied solid-phase extraction by the Sep-Pak Light tC2 cartridge described in the Experimental section for the preparation of blood samples.

The typical chromatograms are shown in Figure 7. There was no impurity peak around oleandrin in the chromatogram of blank bovine plasma. This showed the effectiveness of solid-phase extraction by using a Sep-Pak Light tC2 cartridge and specific fluorescent postcolumn labeling.

**Recovery, linearity, and sensitivity of the method**

The recovery of oleandrin was determined for blank plasma spiked with oleandrin under this study’s extraction procedure. Obtained recoveries of oleandrin from spiked plasma were 98.7% and 88.8%, and relative standard deviation values were 9.42 and 5.33 at 4 and 20 ng/mL, respectively (n = 3).

The fluorescence response to oleandrin in plasma was linear from 2.5 to 250 ng/mL. The equation and \( r^2 \) value were \( y = 8328.8x + 290.94 \) and 0.9994, respectively.

The detection limit of oleandrin in plasma was 1.5 ng/mL (S/N = 3). This postcolumn derivatization method has better sensitivity than UV detection (4) or a precolumn label method (6).

**Delivery of hydrochloric acid**

The peristaltic pump previously used (12) for the delivery of hydrochloric acid limited the choice of flow rate and reactor length. Therefore, several workers pumped concentrated hydrochloric acid indirectly (9,10). In particular, hexane was pumped in order to displace hydrochloric acid out of the chamber. In this study, we used an HPLC pump with a chemically inert pump head to deliver hydrochloric acid. The pump and tubing for hydrochloric acid delivery were rinsed daily with water at the end of operation. Even after several months of operation, there was no sign of any damage in the pump head. This observation indicates the usefulness of the chemically inert HPLC pump to deliver concentrated hydrochloric acid.

**Conclusion**

The proposed method has high sensitivity and specificity, and the instruments used are not as expensive as those of LC–MS. Therefore, this method can be employed as a practical method for the diagnosis of oleander poisoning.

**References**


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