Simultaneous Determination of Cardenolides by Sonic Spray Ionization Liquid Chromatography–Ion Trap Mass Spectrometry—A Fatal case of Oleander Poisoning*

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Abstract

Simultaneous determination of oleandrin and its three related compounds, desacetyloleandrin, oleandrigenin, and gitoxigenin in blood by using liquid chromatography–three-dimensional quadrupole mass spectrometry (LC–3DQMS) system equipped with sonic spray ionization (SSI) interface was conducted. This analyzing method was suitable for all of these compounds except gitoxigenin. The limits of detection of oleandrigenin and desacetyloleandrin from blood were 2 ng/mL and that of oleandrin was 3 ng/mL. The calibration curves for oleandrin, desacetyloleandrin, and oleandrigenin were linear in the range of 5–100 ng/mL. The coefficients of variation of oleandrin, desacetyloleandrin, and oleandrigenin in the blood were satisfactory ranging from 1.6% to 4.1%. This analysis method was applied to a fatal case of oleander poisoning. As a result of liquid chromatography–mass spectrometry (LC–MS) analysis, oleandrin was detected in heart blood and cerebrospinal fluid. Desacetyloleandrin, oleandrigenin, and gitoxigenin were not detected. In order to make identification of oleandrin reliable, LC–MS–MS analysis was performed. The concentrations of oleandrin found in the heart blood and cerebrospinal fluid were 9.8 and 10.1 ng/mL, respectively.

Introduction

Oleander (Nerium oleander L., N. odorum Sol., Apocynaceae) is an evergreen shrub and widely distributed in temperate, subtropical and tropical areas. This plant contains cardenolides, such as oleandrin, oleandrigenin, and desacetyloleandrin (1).

Some human oleander poisoning cases have been reported (2–4). It may be difficult to analyze oleandrin by gas chromatography–mass spectrometry because oleandrin is not stable on heating. There are a few reports on the method of analysis of oleandrin by thin-layer chromatography (TLC) (3), high-performance liquid chromatography (HPLC) combined with a fluorometric detector (5), HPLC combined with an ultraviolet detector (6), liquid chromatography–mass spectrometry (LC–MS) (2,7), and liquid chromatography–tandem mass spectrometry (LC–MS–MS) (8).

There is no available report about oleander metabolism in humans. We confirmed that oleandrin is quickly metabolized to desacetyloleandrin in vivo of rats (9). The sugar unit of oleandrin is thought to be easily removed by digestion. Therefore, we designed a selective and sensitive method to determine oleandrin, desacetyloleandrin, oleandrigenin, and gitoxigenin in human blood using LC–MS and LC–MS–MS, and we applied this method to a suspected case of poisoning due to ingestion of water extract of oleander branches and leaves.

Materials and Methods

Materials

Oleandrin, oleandrigenin, gitoxigenin, and digitoxigenin were purchased from Sigma Chemical Co. (St. Louis, MO). Desacetyloleandrin (230 mg) was obtained by alkaline hydrolysis of oleandrin (500 mg) using 0.01N sodium methoxide in absolute methanol (100 mL, -15°C, 12 h) and purified with silica gel column chromatography and recrystallization. The structure and composition of elements were confirmed by using nuclear magnetic resonance spectrometry and elemental anal-
ysis. Other chemicals and solvents used were of analytical grade or higher. The stock solutions (1 mg/mL) of oleandrin, olean- 

drigenin, desacetyloleandrin, gitoxigenin, and digitoxigenin 

were prepared by dissolving each of them with acetonitrile. 

The solutions were stored at -15°C. Biological fluids were 

stored at -30°C prior to analysis.

**Apparatus**

A model M-8000 Hitachi LC-3DQMS system equipped with 

sonic spray ionization (10) interface and GH-C18(III) column 

(150 mm × 2.1-mm i.d., 5 μm, Hitachi) were used. The column 

temperature was 40°C. The mobile phase was water/methanol 

(4:6, v/v). The rate of flow was 0.2 mL/min. The conditions 

of MS for quantitative analysis are shown in Table I, and the 

conditions of MS-MS for qualitative analysis are shown in 

Table II.

**Extraction procedure**

One-hundred microliters of internal standard (IS) solution 

digitoxigenin, 0.1 μg) and 4 mL of water were added to 1 mL 

of blood. The mixture was extracted with 1-chlorobutane (2 mL, 

3 times). The organic layer was concentrated to dryness at 

40°C under a stream of nitrogen gas. The residue was dissolved 

in 0.5 mL of water/methanol (2:8, v/v) and washed with 

n-hexane (1 mL, 2 times). The aqueous layer was concentrated 

to dryness at 40°C under nitrogen gas stream, and the residue 

was dissolved with 100 μL of mobile phase. After centrifugation 

(15,000 × g, 5 min), 5 μL of supernatant was injected into the 

LC-MS system.

The tests to determine the precision and the accuracy of the 

method were performed by using the cardenolides-free human 

blood which was spiked with oleandrin, desacetyloleandrin, 

oleandrigenin, and gitoxigenin (concentrations of each com- 

pound were 10 and 100 ng/mL).

**Case History**

A 49-year-old woman, who had suffered from schizophrenia 

since she was 27, had attempted suicide several times by taking 

psychotropic drugs. Her mental condition became unstable 

one month before death. By coincidence, she learned about 

oleander poisoning on TV. After seeing this program, she pre- 

pared and drank a water extraction of oleander for the purpose 

of suicide. Thirty minutes later, she had numbness of the 

tongue, nausea, and vomiting. She was admitted to a hospital 

approximately 2 h after the onset of the symptoms.

On admission, her consciousness was clear and her vital 

signs were normal. In spite of intensive therapies, however, ar- 

rhythmias such as atrial fibrillation, ventricular tachycardia, 

and ventricular fibrillation increased, and she died one day 

after admission. An autopsy was not performed. At postmortem 

inspection, heart blood and cerebrospinal fluid samples for tox- 

cological analysis were collected by puncture.

<table>
<thead>
<tr>
<th>Table I. Conditions Set for MS for Quantitative Analysis</th>
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<tr>
<td><strong>Ion source</strong></td>
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<tr>
<td>Shield temperature</td>
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<td>Aperture 1 heater</td>
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<td>Drift voltage</td>
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<td>Micro scan</td>
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<td>Mass defect</td>
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<td>Accumulation time</td>
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<td>Low mass cutoff</td>
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<td>Polarity of detected ion</td>
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<td>Scan range</td>
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<th>Table II. Conditions Set for MS-MS for Qualitative Analysis</th>
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<tr>
<td><strong>MS-1</strong></td>
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<tr>
<td>Ion accumulation</td>
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<td>Scan range (amu)</td>
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<tr>
<td>Low mass cutoff (amu)</td>
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<tr>
<td>Accumulation time (ms)</td>
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<tr>
<td>Accumulation Voltage (V)</td>
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<tr>
<th>Isolation step</th>
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<tr>
<td>Isolation masses (amu)</td>
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<tr>
<td>Low mass cutoff (amu)</td>
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<tr>
<td>Isolation time (ms)</td>
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<td>Isolation voltage (V)</td>
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| **MS-2** | for OLAN* | for DAON | for OLAG | for GITG |
| CID resonance (amu) | 584.00-614.50 | 544.17-570.46 | 444.00-466.00 | 405.88-420.38 |
| Low mass cutoff (amu) | 190 | 200 | 130 | 110 |
| CID time (ms) | 50 | 50 | 50 | 50 |
| CID voltage (V) | 0.188 | 0.170 | 0.185 | 0.140 |

* Abbreviations: OLAN, oleandrin; DAON, desacetyloleandrin; OLAG, oleandrigenin; GITG, gitoxigenin; and CID, collisionally induced dissociation.
Results

Determination of the cardenolides
Under these LC–MS conditions, gitoxigenin, oleandrigenin, digitoxigenin, desacetyloleandrin, and oleandrin were clearly separated and detected at 5.2, 6.4, 8.8, 12.1, and 16.3 min, respectively (Figure 1A). In the mass spectrum of gitoxigenin (Figure 1B), two ion peaks were observed at m/z 413 ([gitoxigenin + Na]⁺) and 395 ([gitoxigenin + Na – H₂O]⁺). In the mass spectra of oleandrigenin (Figure 1C), digitoxigenin (Figure 1D), desacetyloleandrin (Figure 1E), and oleandrin (Figure 1F), the peaks of sodium addition ion were observed at m/z 455 ([oleandrigenin + Na]⁺), 397 ([digitoxigenin + Na]⁺), 557 ([desacetyloleandrin + Na]⁺), and 599 ([oleandrin + Na]⁺), respectively. Therefore, mass chromatograms at m/z 413, 455, 397, 557, and 599 were used for detection of gitoxigenin, oleandrigenin, digitoxigenin, desacetyloleandrin, and oleandrin in LC–MS quantitative analysis. Mass chromatograms of extracts from blank blood and blood containing 10 ng/mL each of oleandrin, desacetyloleandrin, oleandrigenin, and gitoxigenin and 100 ng/mL of IS are shown in Figure 2. There were no interfering peaks on the mass chromatograms of the blank blood. No gitoxigenin peak appeared on the mass chromatogram at the monitoring of m/z 413.

Recovery and limits of detection from human blood
The recovery rates from blood were measured using different concentrations (10 and 100 ng/mL) of these cardenolides, and the limits of detection are shown in Table III. Except for gitoxigenin, the percentage of recovery was 70% or more for each compound. The limits of detection of oleandrigenin, desacetyloleandrin, and oleandrin from blood were 2 ng/mL, and that of oleandrin was 3 ng/mL. In this analyzing method, the recovery of gitoxigenin was low at 21%. Therefore, the limit of detection of gitoxigenin was 30 ng/mL.

Calibration curves
The standard blood samples containing oleandrigenin, desacetyloleandrin, and oleandrin at concentrations 5, 10, 25, 50, 75, and 100 ng/mL were prepared and analyzed using this extraction method. The calibration curves were obtained by plotting the peak-area ratios of oleandrin, desacetyloleandrin, and oleandrin to IS versus each amount of these substances. The calibration curves for each compound were linear in the range of 5–100 ng/mL.
ng/mL. The correlation coefficient of the calibration curves was 0.999 (Figure 3). The coefficients of variation of olean-
drin, desacetyloleandrin, and oleandrigenin in the blood were
satisfactory, ranging from 1.6% to 4.1%.

Application for a fatal oleander poisoning case
We applied this method to the present case. The blood was an-
alyzed using the method described here. The mass chro-
matograms at \textit{m/z} 599, 577, 455, and 397 are shown in Figure
4. Three major peaks at the retention time of 11.2, 13.2,
and 16.4 min were observed on the mass chromatograms. The
peak at 16.4 min in the mass chromatogram at \textit{m/z} 599 was thought
to be oleandrin. To confirm that the peak at 16.4 min in the
mass chromatogram at \textit{m/z} 599 was oleandrin, LC-MS-MS
analysis was carried out. The solvent of the blood extract was
evaporated. After redissolving with 20 μL of mobile phase, 5-μL
aliquot was injected into the LC–MS system. The mass spectra and product ion spectra of oleandrin standard and the peak at
16.4 min of the blood are shown in Figure 5. The ion at \textit{m/z} 599
of the peak at 16.4 min gave product ion peaks at \textit{m/z} 539 and
455. Because the product ion spectrum showed the same pat-
tern as that of oleandrin (Figure 4B), the peak at 16.4 was
identified to be oleandrin. As a result of the quantitative anal-
ysis, the concentration of oleandrin was 9.8 ng/mL in the blood
and 10.1 ng/mL in the cerebrospinal fluid. In LC–MS and
LC–MS–MS analysis for the blood sample of this case, neither
desacetyloleandrin nor oleandrigenin were detected. Although
two other peaks were not able to be identified, they were con-
sidered to come from oleander because they were detectable
also from the extract of oleander branches and leaves.

| Table III. Recovery of Each Cardenolide and Limit of
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<tr>
<th>Detection from Human Blood</th>
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<th>Recovery (%)*</th>
<th>LOD†</th>
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<tr>
<td></td>
<td>10 ng/mL</td>
<td>100 ng/mL</td>
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<tr>
<td>Oleandrin</td>
<td>86.6 ± 7.1</td>
<td>76.7 ± 3.1</td>
</tr>
<tr>
<td>Desacetyloleandrin</td>
<td>69.2 ± 5.9</td>
<td>87.6 ± 1.2</td>
</tr>
<tr>
<td>Oleandrigenin</td>
<td>72.7 ± 4.8</td>
<td>82.3 ± 3.6</td>
</tr>
<tr>
<td>Gitoxigenin</td>
<td>N.D.‡</td>
<td>21.2 ± 1.8</td>
</tr>
<tr>
<td>Digitoxigenin (IS)</td>
<td>N.T.</td>
<td>92.9 ± 0.9</td>
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* \( n = 5 \), mean ± standard deviation.
† Signal-to-noise ratio is 3.
‡ N.D., not detected and N.T., not tested.

Discussion
In this present study, simultaneous determination of olean-
drin and its three related compounds, desacetyloleandrin, ole-
andrigenin, and gitoxigenin, in blood by using LC–3DQMS
system was demonstrated. We chose a sonic spray ionization in-
terface as ion source because these cardenolides are thermal-
able and nonvolatile compounds. Sonic spray ionization is soft
ionization without high voltage. This process does not require
high temperatures; therefore, sonic spray ionization is suit-
able for analyzing unstable compounds. In each mass spec-
trum of oleandrin, desacetyloleandrin, oleandrigenin, and
digitoxigenin (I.S.) standard, only one peak that is molecular ion accompanied with
sodium was observed. In the mass spectrum of
gitoxigenin, two peaks which are molecular
ion accompanied with sodium and the ion
which lost H₂O were observed. It seems that
the cleavage of peak on the mass spectrum ef-
tects sensitivity. The absolute limit of detection
of oleandrin of this method was 0.15 ng. This
was slightly higher than that of the report of
Tracqui et al. (3). They used ionspray atmos-
pheric pressure interface, the results of which
showed four ions' peaks on the mass spectrum
of oleandrin.

A toxicological analysis of oleandrin, de-
cacetyloleandrin, and oleandrigenin in whole
blood and cerebrospinal fluid that were ob-
tained from a cadaver of the suspected case of
oleander poisoning were performed. Olean-
drin was detected and confirmed using the
LC–MS–MS technique. The concentrations
of oleandrin in the blood and the cerebrospinal
fluid were 9.8 ng/mL and 10.1 ng/mL, respec-
tively. This blood level was higher than in a
non-fatal oleander poisoning case (1.1 ng/mL)
(3) and slightly lower than the digoxin con-
centrations reported in fatalities (22 ng/mL
and 30 ng/mL) (11,12). Blum and Rieders (3)

\begin{figure}
  \centering
  \includegraphics[width=\textwidth]{figure3.png}
  \caption{Calibration curves for oleandrin, oleandrigenin, and desacetyloleandrin. The vertical axis shows the peak-area ratios of oleandrin, desacetyloleandrin, and oleandrigenin to digitoxigenin.}
\end{figure}
have reported that the concentration of oleandrin in blood from a fatal case of oleander poisoning was 12 μg/mL. This value is remarkably high compared with the concentration in this case. It seems that they might have measured not only oleandrin, but also some of the components of N. oleander, because the quantitative assay was performed by fluorescence spectrophotometry on the dry extract reconstituted in water/methanol without using the chromatographic technique. Therefore, the cause of death of this case was decided to be oleander poisoning.

As a result of analysis for this present case, only oleandrin could be detected. N. oleander contains oleandrin (0.13% in oven-dried leaves) as its main cardenolide and traces of desacetyloleandrin, oleandrigenin, and gitoxigenin etc. (2). Oleandrin is metabolized to desacetyloleandrin in vivo of rats quickly (10). It was an unexpected result that desacetyloleandrin was undetectable in the blood and the cerebrospinal fluid of a human fatal case of oleander poisoning, although desacetyloleandrin was detected in the biological materials (blood and liver) of the rat to which oleandrin was administered.

In conclusion, the LC-MS method is useful for confirmation of oleander poisoning as described by Tracqui et al. (2). Furthermore, the analysis by using LC–MS–MS has higher qualitative ability and was thought to be useful for the determination of oleander poisoning from rotten biological materials which is often seen in forensic cases.

### Acknowledgments

This study was supported by Grants-in-Aid for Encouragement of Young Scientists (11770234). We are grateful to Dr. F. Abe and Dr. J. Kinjo (Faculty of Pharmaceutical Sciences, Fukuoka University) for their helpful advice, to Dr. J. Tanaka (Faculty of Science, University of the Ryukyus) for measurement of the NMR spectra, and to Prof. T. Nohara and Dr. T. Ikeda (Faculty of Pharmaceutical Sciences, Kumamoto University) for elemental analysis.

### References


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