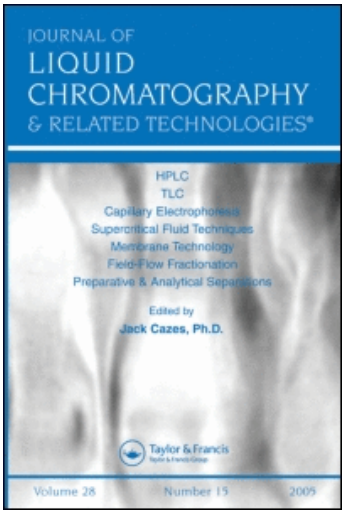


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# HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY– TANDEM MASS SPECTROMETRY OF CARDIAC STEROIDS

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## ABSTRACT

High performance liquid chromatography-tandem mass spectrometry (LS/$MS^n$) utilizing an ion trap mass spectrometer has been used for the analyses of representative cardiac steroids. In the case of bufotoxin having a polar residue (suberoylarginine), the positive-electrospray ionization (ESI) was 50 times more sensitive than the atmospheric pressure chemical ionization (APCI) and provided information concerning the molecular weight as well as the structures of both aglycone and suberoylarginine. The corresponding bufogenin showed 2 times better sensitivity in the positive-APCI than ESI. The mass spectrum of digoxin with the positive-APCI showed fragment ions not only derived from aglycone but also formed by losses of sugars.

The cleavage of the dioxane ring has been observed on the cardiac glycoside having an unusual sugar linkage. Fast atom bombardment mass spectra of these compounds were examined and compared with the data obtained from LC/MS$^n$.

## INTRODUCTION

Cardiac steroids are classified into two groups, namely bufadienolide and cardenolide having a six- or five-membered lactone ring at the 17β-position of the steroid, respectively. The skin secretion of local toads (toad venom), known as "*Ch'an Su*" in China and as "*Senso*" in Japan, has been employed for centuries as galenical cardiac or diuretic preparations. The main cardiotonic constituents in toad venom have been elucidated to be bufadienolide consisting of bufotoxins such as marinobufotoxin (**1**) having a suberoylarginine ester and bufogenins such as marinobufagin (**2**).[1,2] The other bufadienolides such as homologs and analogs of bufotoxin have also been isolated from toad venom and their structures were confirmed by one of the authors (Shimada).[3] Digoxin **(3)** having three sugar moieties at the 3β-position of the steroid belongs to cardenolide and has been widely used for treating congestive heart failure and some other cardiac diseases. Elaeodendroside B (**4**) also belongs to cardenolide and cardiac glycoside but has an unusual sugar linkage (a dioxane-type, six-membered hemiketal ring) which is not cleaved by the usual enzymatic, acid or base treatment;[4] therefore, much interest is focused on its metabolic pathway. Because these cardiac steroids are among the cardiovascular drugs with the narrowest toxic/therapeutic margins and their respective pharmacokinetics differ strongly individually,[5] analyses of these steroids and their metabolites are significant.

Recently, much interest has been focused on high performance liquid chromatography (LC)/mass spectrometry (MS), especially using ion trap MS, for the analysis of biological substances.[6] The method is attractive because of its easy performance for multi-stage tandem MS (MS/MS, MS/MS…MS$^n$), which is highly specific and helpful for the determination and structure elucidation of metabolites in biological fluids.

Here we investigated the analyses of the above cardiac steroids (**1**-**4**) and related compounds (**5**-**9**) using an LC/ion trap mass spectrometer. First, a suitable ionization method using electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) ion source was examined to obtain higher sensitivity. Collisionally induced dissociation (CID) of molecular-related ions (LC/MS/MS) and successive CID of the selected ions (LC/MS/MS/MS) were used for the structure elucidation of these steroids, which was done by activating the precursor ions with 20-30% relative collision energy.

On the other hand, fast atom bombardment (FAB)-MS of the cardenolide is known to provide information concerning not only the molecular weight but also the aglycone and sugar moieties.[7] Here, the negative FAB mode using glycerol as a matrix was also used to examine these cardiac steroids to compare with the data obtained from LC/MS$^n$. High resolution electron ionization (EI)- and FAB-MS were also utilized to confirm some fragment ions observed in LC/MS$^n$.

## EXPERIMENTAL

### Materials and Reagents

Marinobufotoxin (**1**),[8] marinobufagin (**2**),[8] elaeodendroside B (**4**),[4] marinobufagin 3- suberoylhistidine ester (**5**),[9] marinobufagin 3-pimeloylarginine ester (**6**),[8] altoside (**7**)[10], and elaeodendroside A (**9**)[11] were previously isolated or synthesized by one of the authors (Shimada) and his co-workers. Digoxin (**3**) and the liquid matrix for FAB-MS; glycerol, *m*-nitrobenzylalcohol (NBA), dithiothreitol (DTT), thioglycerol (TG), and magic bullet [DTT:dithioerythritol (3:1)] were purchased from Tokyo Kasei (Tokyo, Japan). Cymarin (**8**) was obtained from Sigma (St. Louis, Mo, U.S.A.). All other reagents and solvents were of analytical grade.

### Instrumentation and HPLC Conditions

The LC/MS$^n$ was performed using a Finnigan MAT LCQ (San Jose, CA, USA) connected to a JASCO PU-980 (Tokyo) chromatograph. A YMC Pack Pro C18 (5 μm, 150 x 3.0 mm I.D.)(YMC, Kyoto, Japan) was used at a flow rate of 0.4 mL min$^{-1}$. Conditions of detection were as follows: ESI - the spray needle voltage was 5 kV, the heated capillary temperature, sheath gas flow rate, and auxiliary gas flow rate were set at 200°C, 70 units and 20 units, respectively. The capillary voltage was 3 or -3 V, and the tube lens offset was 30 or -30 V in the positive- or negative-ion mode, respectively. APCI - the spray needle voltage was 6 kV, the heated capillary temperature and sheath gas flow rate were set at 225°C and 80 units with a vaporizer temperature of 400°C. The capillary voltage was 3 or -3 V, and the tube lens offset was 30 or -30 V in the positive- or negative-ion mode, respectively. Nitrogen gas was used as the sheath gas, and CID was performed in the trap with helium gas as the collision gas.

The EI mode analyses were performed with a JEOL JMS-600W double-focusing (EB geometry) mass spectrometer with a JEOL MS-MP09 data system

(Tokyo) which was operated with a filament current of 300 μA and an electron energy of 70 eV.

The ion source was held at 200°C, the temperature of the probe increased from 60°C to 300°C at a rate of 128°C $min^{-1}$ and finally it was held at 300°C. For the high resolution-EI-MS, the resolution was 3000 and perfluorokerosene was used as a standard.

The FAB mode analyses were performed with a JEOL JMS-SX102 double-focusing (BE geometry) mass spectrometer with a JEOL complement data system. The fast atom, accelerating voltage, and the gun emission current were xenon, 3 kV, and 10 mA, respectively. The resolution for low- and high-resolution-FAB-MS were 1000 and 3000, respectively. One microliter (2 μL in the case of **3**) of methanol solutions of the samples was mixed with 1 μL of matrix; analyses were then carried out at a 12 sec cycle time within a 10-1000 mass range.



The samples were introduced into the ion source using a direct inlet system in the EI- and the FAB-MS.

## RESULTS

The LC/$MS^n$ and the FAB mass spectra of **1-4** together with related compounds (**5-9**) were examined as described below. The EI mass spectra were also examined as the occasion demanded. In the FAB-MS, although the positive-ion mode and other matrices (NBA, DTT, TG, and magic bullet) were tried, the negative-ion mode utilizing glycerol as the matrix gave the best responses, in which glycerol cluster ions were subtracted. With each compound, the following ion species were observed.

### Marinobufotoxin (1)

First, the LC/APCI-MS was carried out using MeOH-$H_2O$ (5:2, v/v, $t_R$ 3.3 min) as a mobile phase. The fragment ion (*m/z* 401) relating to the aglycone was observed together with the molecular-related ion ($[M+H]^+$) at *m/z* 713 in the positive-ion mode, and the negative-ion detection gave only a fragment ion corresponding to the aglycone (*m/z* 399). On the other hand, in the ESI-MS, the molecular-related ions at *m/z* 713 and 711 ($[M-H]^-$) were obtained in the positive- and the negative-ion mode, respectively, but no fragment ions were observed (Figure 1a, b).

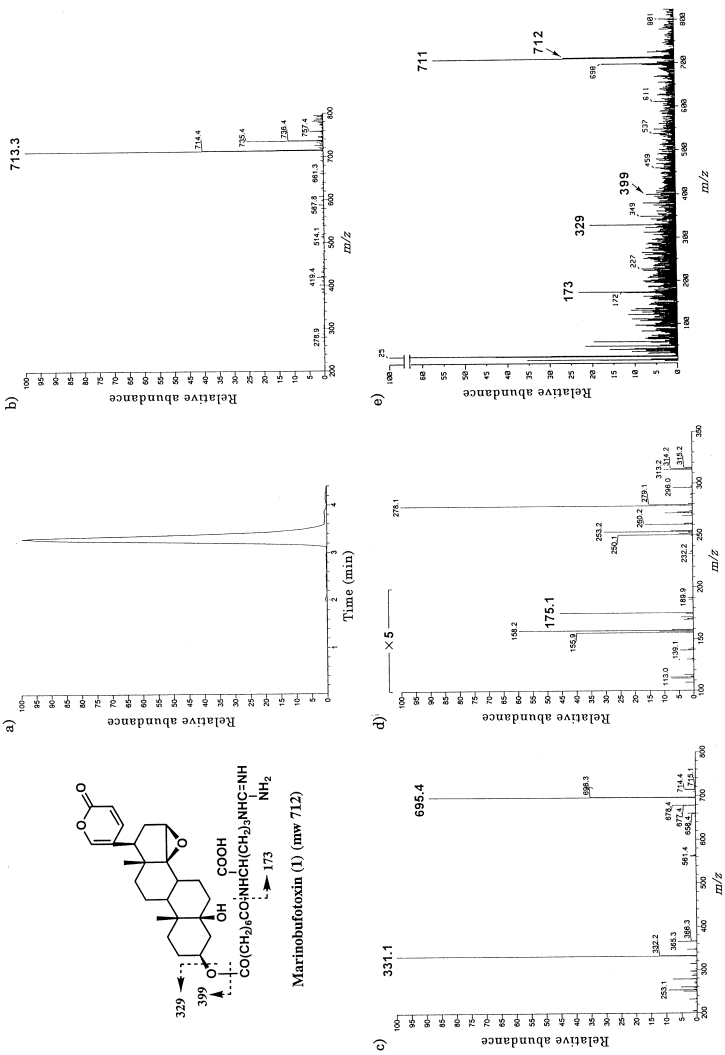



**Figure 1.** LC/ESI-MS chromatogram, ESI-MS$^n$ spectra, and FAB mass spectrum of marinobufotoxin (**1**). a) LC/ESI-MS chromatogram: *m/z* 713 was monitored after *m/z* 200-800 was scanned. b) Positive-ESI mass spectrum. c) Product ion mass spectrum (Positive-ESI/MS/MS): precursor ion; *m/z* 713, relative collision energy; 30%. d) Product ion mass spectrum (Positive-ESI/MS/MS/MS): precursor ion; *m/z* 713 and then 331, relative collision energy; 30% each. e) Negative-FAB mass spectrum.

**Table 1**

**Comparison of Intensity of Base Ions of Marinobufotoxin (1) in Various Ionization Modes**

| Ionization Mode | Mobile Phase ($t_R$) MeOH-$H_2O$ (5:2, v/v) (3.3 min) | MeCN-$H_2O$ (3:2, v/v) (3.6 min) | MeOH-10 mM $AcONH_4$ (5:2, v/v) (3.5 min) |
|---|---|---|---|
| **Positive-ESI** | | | |
| Base ion (*m/z*) | 713 $[M+H]^+$ | 713 | 713 |
| Intensity (%)[a] | 100 | 73.9 | 30.6 |
| **Negative-ESI** | | | |
| Base ion (*m/z*) | 711 $[M-H]^-$ | 711 | 711 |
| Intensity (%) | 9.9 | 9.5 | 3.4 |
| **Positive-APCI** | | | |
| Base ion (*m/z*) | 401 [aglycone + H]$^+$ | N. E.[b] | N. E. |
| Intensity (%) | 1.8 | | |
| **Negative-APCI** | | | |
| Base ion (*m/z*) | 399 [aglycone-H]$^-$ | N. E. | N. E. |
| Intensity (%) | 0.1 | | |

[a] 40 ng of **1** was injected into LC/MS in each mode. The peak area of the base ions on the LC/MS chromatogram was measured, and the positive-ESI mode using MeOH-$H_2O$ was taken as 100%. [b] Not examined.

The sensitivity of each method was compared using the peak area of the base ions measured by selected ion monitoring (SIM) (Table 1). The largest peak area was obtained in the positive-ESI-MS, which was about 10 and 50 times better than the negative-ESI-MS and the positive-APCI-MS, respectively. We then examined the influence of the mobile phase, that is, other organic modifiers or salts for the sensitivity in positive-ESI-MS. When MeCN was used instead of MeOH as an organic modifier [MeCN-$H_2O$ (3:2, v/v), $t_R$ 3.6 min], only the ion at *m/z* 713 was observed similarly, but its intensity was decreased to about 70%. Furthermore, contrary to expectations, the addition of $AcONH_4$ [MeOH-10 mM $AcONH_4$ (5:2, v/v), $t_R$ 3.5 min] gave only a protonated ion but not an adduct ion ($[M+NH_4]^+$) and reduced the intensity to about 30%. Based on these results, the following analyses with $LC/MS^n$ were performed using MeOH-$H_2O$ (5:2, v/v) as the mobile phase.

The CID of *m/z* 401 $[aglycone+H]^+$ with 30% relative collision energy in the positive-APCI gave a series of fragment ions at *m/z* 383 $[401-H_2O]^+$, *m/z* 365 $[401-2H_2O]^+$, and *m/z* 347 $[401-3H_2O]^+$ in which losses of water were observed.

On the other hand, when the ion at *m/z* 713 $[M+H]^+$ was selected as the precursor ion, the product ions concerning suberoylarginine at *m/z* 331 together with the dehydrated ion at *m/z* 695 were observed. These product ions were also observed in the positive-ESI-MS/MS (Figure 1c). The analog of **1**, marinobufagin 3-suberoylhistidine ester (**5**), having histidine instead of arginine as an amino acid residue and the homolog of **1**, marinobufagin 3-pimeloylarginine ester (**6**), in which the suberoyl group is replaced with a pimeloyl group, were also investigated under the same conditions as **1**. In consequence, **5** and **6** gave the product ions at *m/z* 312 [suberoylhistidine+H]$^+$ and *m/z* 317 [pimeloylarginine+H]$^+$ in the positive-ESI-MS/MS, respectively. Furthermore, in the negative-ESI-MS/MS, the corresponding ion was observed at *m/z* 329, 310 and 315 from **1**, **5** and **6**, respectively.

Subsequent CID of these ions, that is, *m/z* 331, 312 and 317 from **1**, **5** and **6**, in the positive-ion mode gave product ions at *m/z* 175, 156 and 175, respectively, which were inferred to be amino acid residue, arginine and histidine (Figure 1d). The product ions indicating amino acid residue (*m/z* 173, 154 and 173 from **1**, **5** and **6**, respectively) were similarly obtained in the negative-ESI-MS/MS/MS.

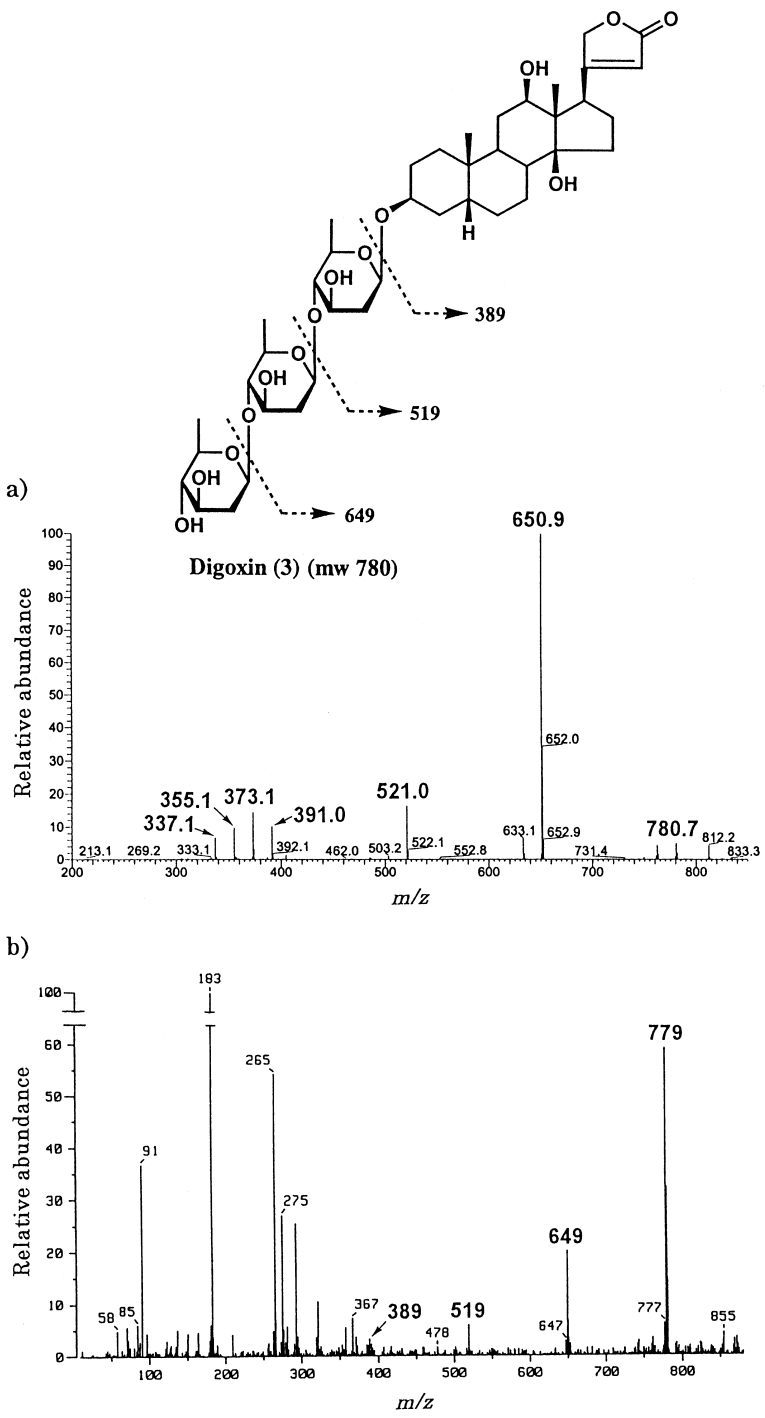
The negative-FAB mass spectrum as shown in Figure 1e presented the ions relating to molecular weight at *m/z* 712 $[M]^-$ and 711 $[M-H]^-$ with strong intensity, as well as characteristic ions concerning the aglycone moiety at *m/z* 399, suberoylarginine at *m/z* 329 and arginine at *m/z* 173. These data are compatible with those obtained from the above LS/MS$^n$.

In order to identify the ion at *m/z* 173 more reliably, the high resolution-FAB-MS was performed utilizing glycerol as a standard. As a result, it was proven that the product ion at *m/z* 173 was derived from arginine [*m/z* 173.1064 (Calcd for $C_6H_{13}O_2N_4$: 173.1039)].

**Marinobufagin (2)**

The LC/MS$^n$ of **2** was performed using MeOH-$H_2O$ (5:2, v/v, $t_R$ 3.8 min) as a mobile phase, because the use of MeCN and $AcONH_4$ caused lower sensitivity. Both the ESI- and the APCI-MS gave ions relating to molecular weight at *m/z* 401 $[M+H]^+$ in the positive-ion mode, and the latter method was superior to the former in sensitivity (about 2 times). The negative-ESI- and APCI-MS gave the molecular-related ion at *m/z* 399, whose intensity was about one-tenth that in the positive-ion mode.

The product ion mass spectrum of the ion at *m/z* 401 in the APCI-MS showed the ions formed by losses of water at *m/z* 383 $[401-H_2O]^+$, 365 $[401-2H_2O]^+$, 347 $[401-3H_2O]^+$. This result could be expected from the data for the APCI-MS of **1**, because **2** was the aglycone of **1**. The EI-MS also gave the fragmentations at *m/z* 382, 364, and 346 $[M-nH_2O]^+$.[12] Although **2** has only two



hydroxy groups, these mass spectra showed the loss of three water molecules. The high resolution-EI-MS showed that the ion at *m/z* 346 was derived from cleavage of the epoxy ring and successive loss of water [*m/z* 346.1950 (Calcd for $C_{24}H_{26}O_2$: 346.1933)].

**Digoxin (3)**

Mobile phases were MeOH-10 mM $AcONH_4$ (5:2, v/v, $t_R$ 3.2 min) for the ESI mode and MeOH-$H_2O$ (5:3 v/v, $t_R$ 6.1 min) for the APCI mode. The ESI-MS gave only adduct ions, $[M+NH_4]^+$ at *m/z* 798 and $[M+AcO]^-$ at *m/z* 839 in the positive- and the negative-ion mode, respectively. The product ion mass spectra of $[M+NH_4]^+$ showed a fragment ion at *m/z* 651 [digoxigenin bisdigitoxoside (bisglycoside)+H]$^+$ as a base ion together with a protonated ion at *m/z* 781 with a weak intensity.

Subsequent CID of the ion at *m/z* 651 led to sequential losses of the glycoside moieties by cleavage of the ether bonds between the glycosidic oxygen and the anomeric carbon[13] to give the ion at *m/z* 521 [monoglycoside+H]$^+$ besides the ions relating to the aglycone at *m/z* 391 and 373 $[391\text{-}H_2O]^+$. On the other hand, in the negative-ESI-MS/MS, only a deprotonated ion at *m/z* 779 was observed, which gave the following product ions by CID (MS/MS/MS); *m/z* 649 [bisglycoside-H]$^-$, 605, 475 [605-glycoside moiety]$^-$, 345 [605-two glycoside moieties]$^-$.

The positive-APCI-MS of **3** gave an ion at *m/z* 651 as a base ion besides the ions at *m/z* 781, 521, 391, 373, 355 $[391\text{-}2H_2O]^+$, and 337 $[391\text{-}3H_2O]^+$ without CID (Figure 2a). Similar results were obtained from altoside (**7**) and cymarin (**8**) (Figure 3), which indicated that the APCI-MS was very useful for identification of the aglycone and sugar moiety on steroid glycosides. Incidentally, in the APCI-MS of **7**, a fragment ion indicating dehydrated aglycone rather than aglycone itself was observed. The FAB-MS spectra of **3** presented the characteristic ions at *m/z* 779 [M-H]$^-$, 649 [bisglycoside-H]$^-$, 519 [monoglycoside-H]$^-$, and 389 [aglycone-H]$^-$ (Figure 2b).

**Elaeodendroside B (4)**

Based on the results of digoxin (**3**), the LC/MS analysis of **4** was performed with the positive-APCI mode using MeOH-$H_2O$ (5:2, v/v, $t_R$ 3.3min) as a mobile phase. In this method, the protonated ion at *m/z* 517 was observed as a base ion, and the following fragment ions were observed with a relative

**Figure 2 (left)**. LC/APCI and FAB mass spectra of digoxin (**3**). a) Positive-APCI mass spectrum. b) Negative-FAB mass spectrum.

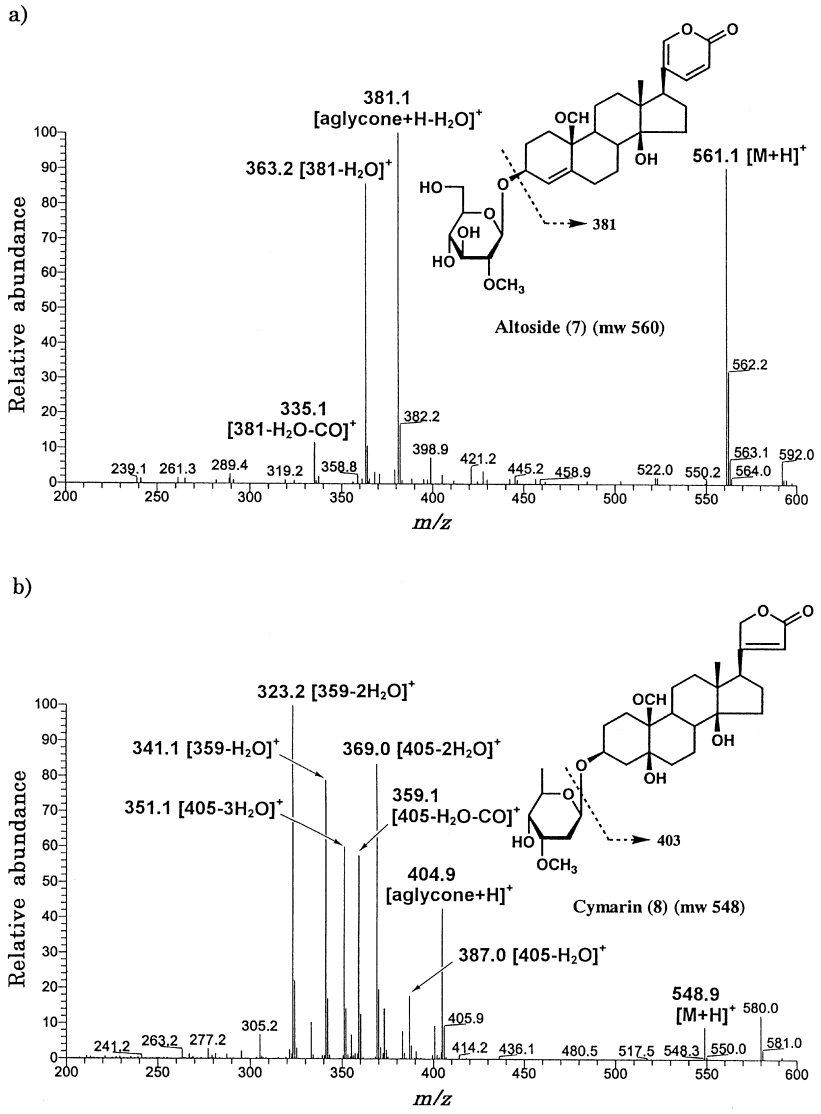
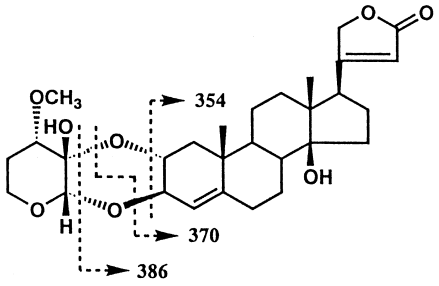


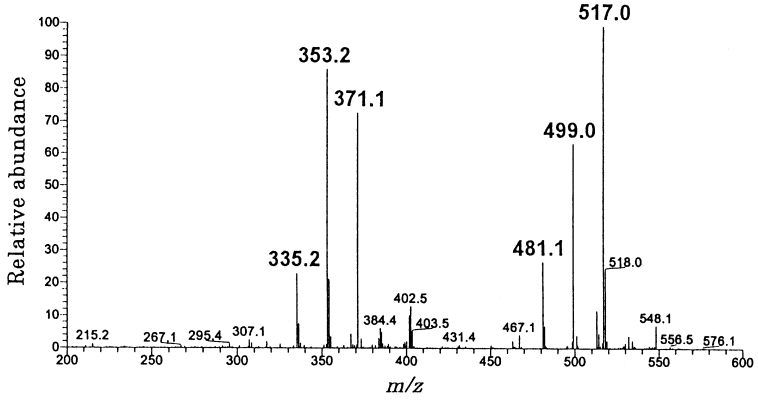

**Figure 3**. LC/APCI mass spectra of altoside (**7**) and cymarin (**8**). Mobile phase; MeOH-$H_2O$ (5:3, v/v), $t_R$ of **7** and **8**; 2.7 and 4.2 min, respectively.

**Figure 4 (right).** LC/APCI and FAB mass spectra of elaeodendroside B (**4**). a) Positive-APCI mass spectrum. b) Negative-FAB mass spectrum.

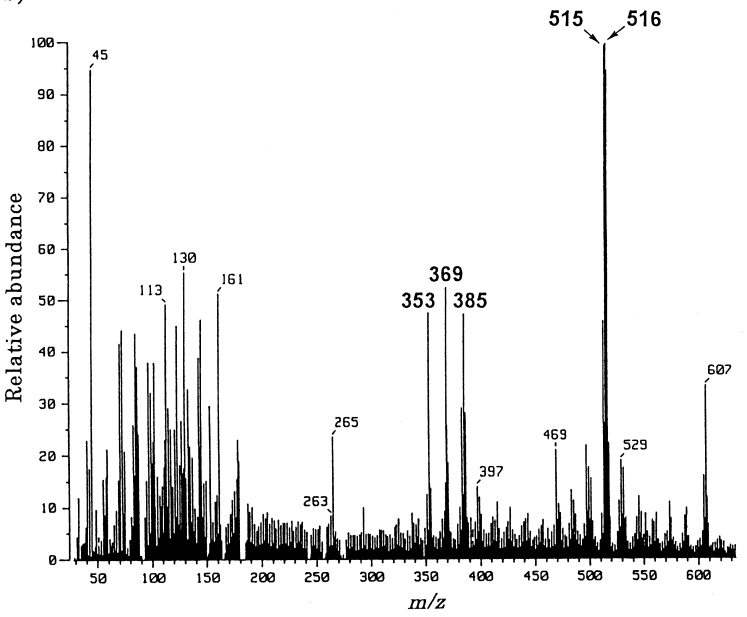



**Elaeodendroside B (4) (mw 516)**

a)




b)



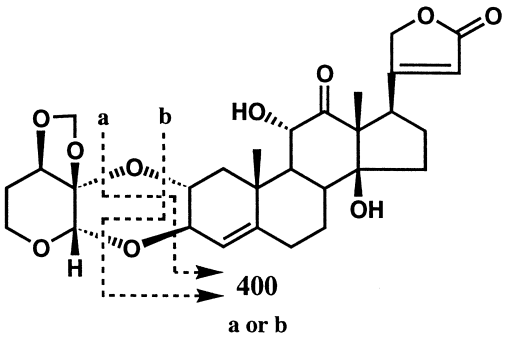



**Figure 5**. Structure of elaeodendroside A (**9**).



strong intensity; *m/z* 499 $[517\text{-}H_2O]^+$, 481 $[517\text{-}2H_2O]^+$, 371, 353 $[371\text{-}H_2O]^+$, and 335 $[371\text{-}2H_2O]^+$ (Figure 4a). As mentioned in the introductory section, **4** has a dioxane-type six-membered hemiketal ring, which is not cleaved by the usual enzymatic, acid or base treatment.[4] However, the ion at *m/z* 371 was inferred to be derived from the cleavage of it. In order to clarify this point, we performed the high resolution-EI-MS of the fragment ions at *m/z* 370 and 354, which were observed in the EI-MS spectrum. Consequently, it was clarified that these ions came from the cleavage of the hemiketal ring as shown in Figure 4 [*m/z* 370.2131 (Calcd for $C_{23}H_{30}O_4$: 370.2144), *m/z* 354.2207 (Calcd for $C_{23}H_{30}O_3$: 354.2195)]. Although it is possible that a positional isomer having a hydroxy group at the 2- or 3-position is formed, the former was expected to be formed preferentially because an allyl radical is more stable.

In addition, the FAB mass spectra also presented the ions relating to the cleavage of the ring with three patterns, *m/z* 385, 369, and 353, besides molecular-related ions at *m/z* 516 $[M]^-$ and 515 $[M\text{-}H]^-$ (Figure 4b). Incidentally, elaeodendroside A (**9**), whose sugar linkage was also a dioxane-type six-membered ring having a methylenedioxygroup instead of a hemiketal group (Figure 5), gave only a deprotonated and a dehydrated ion in the negative-FAB-MS. On the contrary, cleavage of the ring (*m/z* 401) was observed in the APCI-MS.

## DISCUSSION

Both the LC/ESI- and APCI-MS exhibited molecular-related ions, which were useful for determination of molecular weight, but the former gave fewer fragment ions than the latter. From these data, it is considered that the ESI-MS is suitable for quantification. On the other hand, the APCI-MS gave more information about the structure without CID. However, compounds such as marinobufotoxin (**1**) did not provide a sufficient abundance of ions in the APCI-

MS, and its LC/$MS^n$ analysis was performed in the ESI mode. In steroid glycosides, the APCI-MS gave information about aglycone and sugar moieties; the sequential losses of sugar in digoxin (**3)** and the cleavage of the hemiketal ring in elaeodendroside B (**4**). These data suggested that the ESI-mode is suitable for more polar compounds. Furthermore, the intensity of the obtained ion was influenced by the ionization mode (positive or negative) and the organic modifier or salt of the mobile phase, and in the cases of **1** and **2**, the use of MeOH without $AcONH_4$ provided better sensitivity.

The $MS^n$ gave a series of characteristic ions to provide structural information, that is, the aglycone and substituted groups. Fatty acid-amino acid moieties and amino acid residues of bufotoxin, such as **1**,**5**, and **6** could be determined by the ESI-MS/MS and the ESI-MS/MS/MS, respectively. Although the sugar moieties were not determined directly, they could be identified by the difference in mass units between the precursor and the product ions in **3**. However, the fragmentation occurred only at ester, amido, and ether bonds, that is, a carbon-hetero atom bond and a carbon-carbon bond hardly cleaved. One of the reasons for this phenomenon is that the LC/$MS^n$ was performed at low energy. Therefore, in compounds such as marinobufagin (**2**), only losses of water were observed and sufficient information about its structure could not be obtained. On the contrary, the EI-MS gave more fragment ions and was useful for structural analysis for unconjugated steroids.

The FAB-MS presented molecular-related ions besides fragment ions as well as those observed in the LC/$MS^n$. These data indicate that the method called soft ionization containing ESI-, APCI- and FAB-MS gives similar ions.

In conclusion, we examined the usefulness of LC/$MS^n$ for the analyses of cardiac steroids. The results obtained in this study showed that the method will be useful for identification of unknown steroid conjugates obtained from biological fluids.



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