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LC/MS/MS Analyses of an Oleander Extract for Cancer Treatment

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An HPLC/MS/MS method has been developed for the characterization and quantification of the cardiac glycosides oleandrin, odoroside, neritaloside and the aglycone oleandrigenin, all contained in a patented-hot-water extract of *Nerium oleander* L (Anvirzel). Qualitative analysis of such extracts was achieved using a hybrid tandem quadrupole time-of-flight (QqTOF) mass spectrometer. Collision-induced dissociation (CID) mass spectra of oleandrin, oleandrigenin, odoroside, and neritaloside were obtained with greater than 5 ppm mass accuracy and resolution routinely in excess of 8000 (fwhm). The detection limit for oleandrin of 20 pg (injected) was realized when the precursor-to-product ion transition, *m*/*z* 577 → 373, was monitored. We have also applied the analytical method to the determination of oleandrin, oleandrigenin, neritaloside, and odoroside in human plasma following an intramuscular injection of Anvirzel.

Nerium oleander L. is an ornamental plant from the Apocynacea family that is widely distributed in the Mediterranean, subtropical Asia, and the southwestern United States. Although used principally as an ornamental bush, its medicinal and toxicological properties have been recognized for some time. Although accidental or intentional ingestion of this plant has contributed to numerous incidences of poisoning,[1] water and lipid extract preparations both derived from oleander continue to be used as folk remedies for the treatment of a wide variety of maladies and conditions, including abscesses, corns, asthma, dysmenorrhea, eczema, epilepsy, herpes, malaria, psoriasis, ringworm, scabies, sores, warts, and certain tumors.[2−4] Recent unpublished preclinical and clinical uses of Anvirzel, a proprietary hot water extract of oleander leaves currently under development for the treatment of cancer, viral infections, and skin disorders, have suggested positive immune modulatory as well as cytotoxic properties.[5−7]

Although the presence and identification of specific toxic compounds in organic solvent extracts of oleander have been reported,[8−13] knowledge of cardiac glycosides, as well as other plant constituents contained within water-based extracts, is poorly documented. A single brief report indicates that water-soluble extracts of oleander contain polysaccharides with immune-modulatory potential, but no further attempts were made to identify any protein or nonpolar components.[14] Recently, analytical methods for the identification and quantitation of oleandrin in human biological matrixes has been reported, using TLC,[15] HPLC,[16] and LC/MS techniques.[17,18] A more selective, specific, and sensitive technique that uses product-ion scanning via triple-stage quadrupole mass spectrometry has been reported by Henion et al., wherein it was demonstrated that oleandrin and oleandrigenin could be identified at concentrations as low as 30 ppb in a control human liver.[19] Although useful for specific determination of oleandrin and the structurally related compound oleandrigenin, these methods have limited utility for detection of multiple oleander glycosides in human plasma.

The purpose of the present study was to explore the composition of specific compounds (oleandrin, oleandrigenin, odoroside, and neritaloside, see Figure 1) in Anvirzel so that appropriate analytical assays could be developed and applied to the pharmaceutical production and quality control of this botanical product. In addition, we have developed a reliable quantitative analytical assay for the determination of oleandrin, oleandrigenin, odoroside, and neritaloside in human plasma, using mass spectrometry techniques that will be utilized in the upcoming Phase I trial of this novel therapeutic plant extract.

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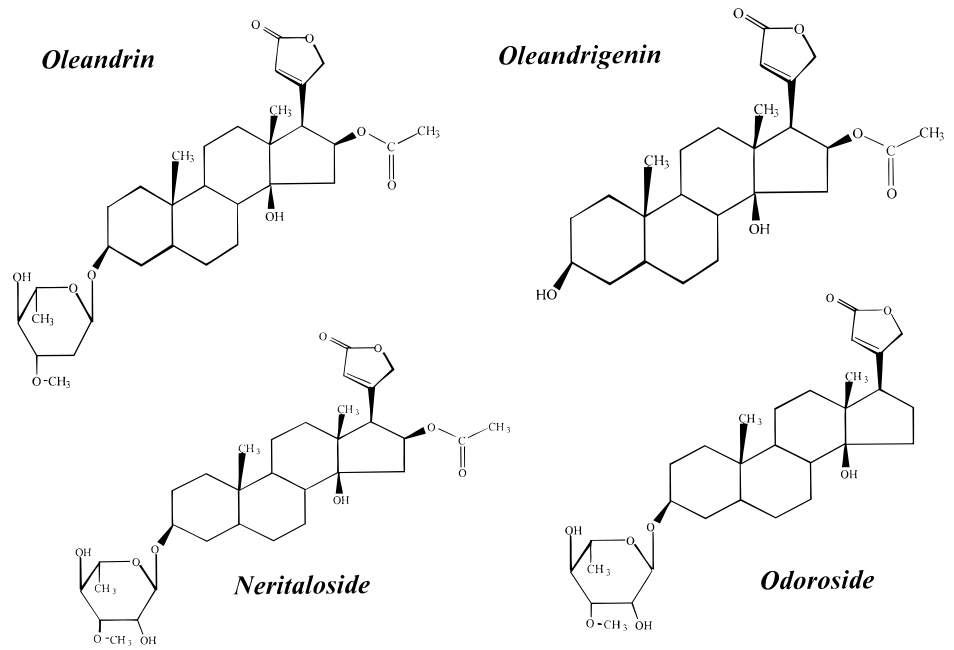

Figure 1. Structures of cardiac glycosides in the oleander extract product Anvirzel.

EXPERIMENTAL SECTION

Materials and Reagents. Oleandrin and Ouabain were obtained from the Sigma Chemical Co. (St. Louis, MO). An oleander extract (used in the preparation of Anvirzel) was provided by Ozelle Pharmaceuticals (San Antonio, Texas). HPLC grade reagents were used for all biological extractions and preparation of standard solutions.

Solid-Phase Extraction (SPE). SPE was used to isolate analytes of interest from human plasma and oleander powder. Ouabain was chosen as an internal standard and was diluted to a working concentration of 1 ng/μL. The plasma samples (1.0−2.5 mL) were loaded onto prepared tC_{18} extraction cartridges (Waters Assoc., Milford, MA). The samples were washed with 2 mL of distilled water, and the analytes were eluted with 2 mL of methanol. The eluents were evaporated to dryness at room temperature under nitrogen and reconstituted in 200 μL of 1:1 acetonitrile/water (v/v) containing 0.1% formic acid. Oleander samples (1 mg oleander extract/milliliter) were also extracted on tC_{18} columns; the nonpolar compounds were eluted with ethyl acetate and were dried under nitrogen. The samples were reconstituted in 200 μL of 1:1 acetonitrile/water (v/v) containing 0.1% formic acid; aliquots (20-μL) were subsequently analyzed. The SPE recovery of ouabain was 90%.

HPLC. All separations were performed at a column temperature of 40 °C with a Waters NovaPak 4-μm C_{18} (2.1 × 150 mm) column operated at a mobile phase flow rate of 300 μL/min. A binary mobile phase consisting of (A) acetonitrile and (B) 0.1% formic acid (aq) was ramped linearly from 10 to 70% A over 18 min.

Mass Spectrometry. QqTOF. The QqTOF instrument used in the current investigation was a prototype constructed from a PE-Sciex API 365 triple-stage quadrupole (Concord, ON, Canada) platform with an orthogonally oriented TOF mass analyzer substituted for quadrupole 3 (Q3). Details of the QqTOF instrumental configuration have been presented elsewhere.[20]

Triple-Stage Quadrupole. All acquisitions were obtained using a PE-Sciex API 3000 triple-stage quadrupole mass spectrometer operated in a multiple reaction monitoring (MRM) mode. Tandem mass spectra were generated at a collision energy of ~15 eV using a N_2 target gas, contained in the collision cell at a pressure of ~6 mTorr.

RESULTS AND DISCUSSION

Qualitative Analysis of Oleander. The electrospray ionizations of the glycosylated cardenolides and aglycone analogues were both characterized by the predominant formation of the $[M + H]^+$ pseudomolecular ion. The biologically active component of oleander, oleandrin, was successfully chromatographed with a retention time (t_r) of 7.4 min, as illustrated in Figure 2 (upper trace). The corresponding QqTOF product-ion spectrum (lower trace, single-point internal recalibration from a residual precursor ion) was characterized by the predominant fragment ions of m/z 517.3166, 433.2595, and 373.2365 which coincided to the suspected loss of neutral acetic acid (CH_3CO_2H), the terminal monosaccharide residue, and both terminal monosaccharide and CH_3CO_2H moieties, respectively. Theoretical m/z ratios for the resultant product ions derived from the above three dissociation pathways are 517.3165 ($C_{30}H_{45}O_7$), 433.2590 ($C_{25}H_{37}O_6$), and 373.2379 ($C_{23}H_{33}O_4$), as results measured mass accuracies of 0.19, 1.10, and 3.75 ppm, respectively. A maximum variation across the product-ion mass range of 3.75 ppm, as measured for the product ion formed via the consecutive loss of monosaccharide and CH_3CO_2H, demonstrates the ability to obtain mass accuracies <5 ppm via single-point internal recalibration. However, it can also be observed that the errors obtained for the minor fragment ion (m/z 145) are greater than 5 ppm. This is probably due to noise because of their low intensity in the spectrum.

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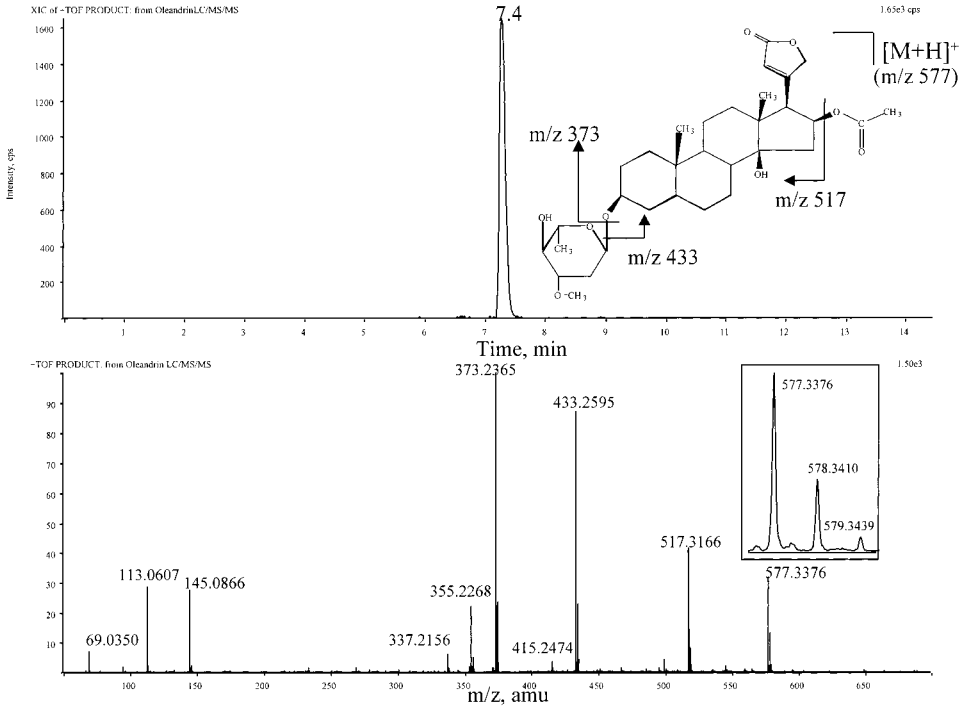

Figure 2. LC/MS/MS spectrum of oleandrin (1 ng/μL) obtained from QSTAR analyses. The selected product-ion current profile for oleandrin is shown in the top chromatogram. The CID spectrum of oleandrin obtained from scanning $[M + H]^+$ (*m/z* 577) is shown in the bottom figure.

Table 1. Accurate Mass Data for Oleander Glycosides

glycoside	nominal mass	formula and calculated mass	experimental mass	err (ppm)
neritaloside	161	$C_7H_{13}O_4$, 161.0814	161.0821	4.4
	373	$C_{23}H_{33}O_4$, 373.2379	373.2389	2.7
	433	$C_{25}H_{37}O_6$, 433.2590	433.2607	3.9
	533	$C_{30}H_{45}O_8$, 533.3114	533.3137	4.3
odoroside	161	$C_7H_{13}O_4$, 161.0814	161.0830	9.9
	375	$C_{23}H_{35}O_4$, 375.2535	375.2539	1.0
oleandrin	145	$C_2H_{13}O_3$, 145.0851	145.0862	7.5
	373	$C_{23}H_{33}O_4$, 373.2379	373.2376	0.8
	433	$C_{25}H_{37}O_6$, 433.2590	433.2610	4.6
	517	$C_{30}H_{45}O_7$, 517.3165	517.3185	3.2
oleandrigenin	373	$C_{23}H_{33}O_4$, 373.2379	373.2381	0.5

On the basis of the fragmentation pattern elucidated from the standard oleandrin, we expected that other cardiac glycosides contained in the oleander extract would exhibit analogous behavior (i.e., the production of an aglycone as well as loss of acetic acid) under similar dissociative conditions. Because of the resolving power of the TOF analyzer, it is possible to accurately measure the masses of the fragment ions using one ion as internal calibrate. In this approach, the unknown cardiac glycosides in the oleander extract can be identified. Tandem mass spectrometric analysis of each component provided the total ion chromatograms presented in Figure 3 and the corresponding product ions for these oleander glycosides listed in Table 1. In Table 1, only the major product ions are listed, several water losses from the product ions are also evident. On the basis of these precursor and product ion *m/z* ratios and better than 5 ppm mass accuracy (except for the minor fragment ions of *m/z* 145 and 161), the unknown compounds in oleander extract were tentatively identified as neritaloside, oleandrigenin, odoroside, and oleandrin, respectively.

The product-ion current profile shown in Figure 3 derived from the precursor ion of *m/z* 577 indicates the presence of three nominally isobaric components. The two components that elute at 6.7 and 7.2 min have identical product-ion spectra to that of oleandrin. Thus, these two compounds are likely cardiac glycosides that are structural isomers of oleandrin, and most likely are cryptograndoside-A and nerigoside, both of which have previously been identified in extracts of oleander.[21]

Quantitative Analysis of Oleander. Having confirmed, by product-ion accurate mass assignment, the presence of neritaloside, oleandrigenin, odoroside, and oleandrin in oleander extracts, the next stage of the investigation was to develop a quantitative protocol for these target analytes that could readily be applied in a pharmacokinetic study. To achieve the optimal method detection limits (MDLs), a triple-stage quadrupole mass spectrometer operated in the multiple-reaction monitoring (MRM) mode was optimized for the precursor → product ion transitions of *m/z* 593 → 373, 433 → 373, 535 → 375, and 577 → 373 for neritaloside, oleandrigenin, odoroside, and oleandrin, respectively. Figure 4 shows that 1 ng/mL of oleandrin in human plasma can be detected, and the signal-to-noise ratio is better than 3/1.

The response as a function of concentration could only be measured for oleandrin because it was the only cardiac glycoside available commercially. Thus, a five-point calibration curve with a dynamic range that extended 4 orders of magnitude was constructed, using ouabain as an internal standard (monitoring the 585 → 513 transition); data were obtained in triplicate for concentration. The instrumental detection limit for oleandrin was 20 pg (injected), and the peak area ratio of oleandrin to ouabain was linear with a correlation coefficient $r^2 > 0.9998$.

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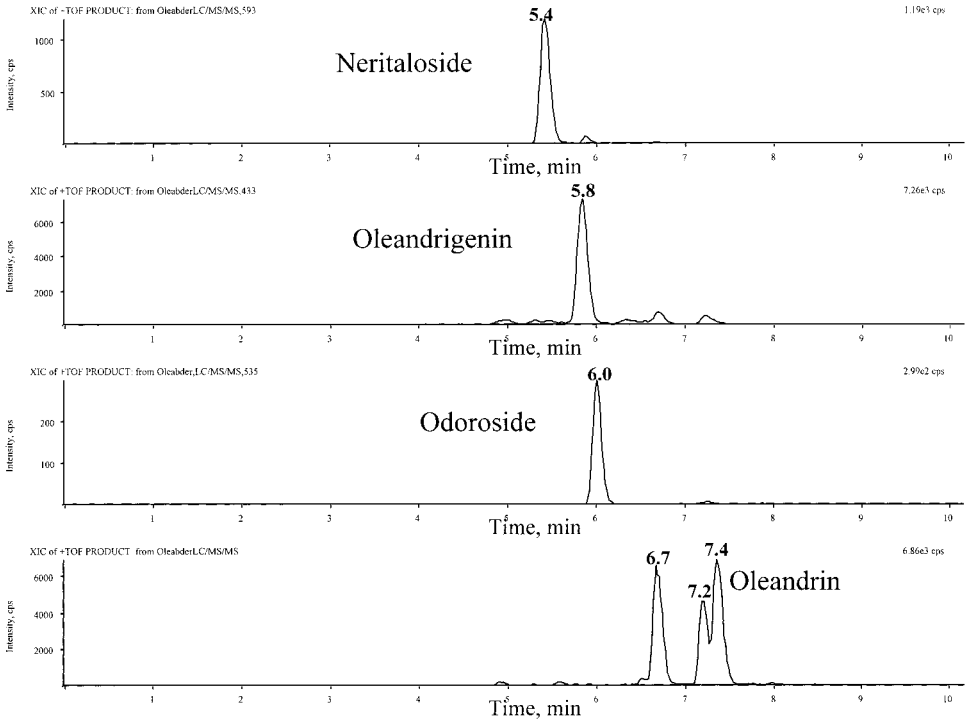

Figure 3. LC/MS/MS chromatograms of neritaloside, oleandrigenin, odoroside, and oleandrin from a 100 μg/mL oleander extract obtained from QSTAR analyses.

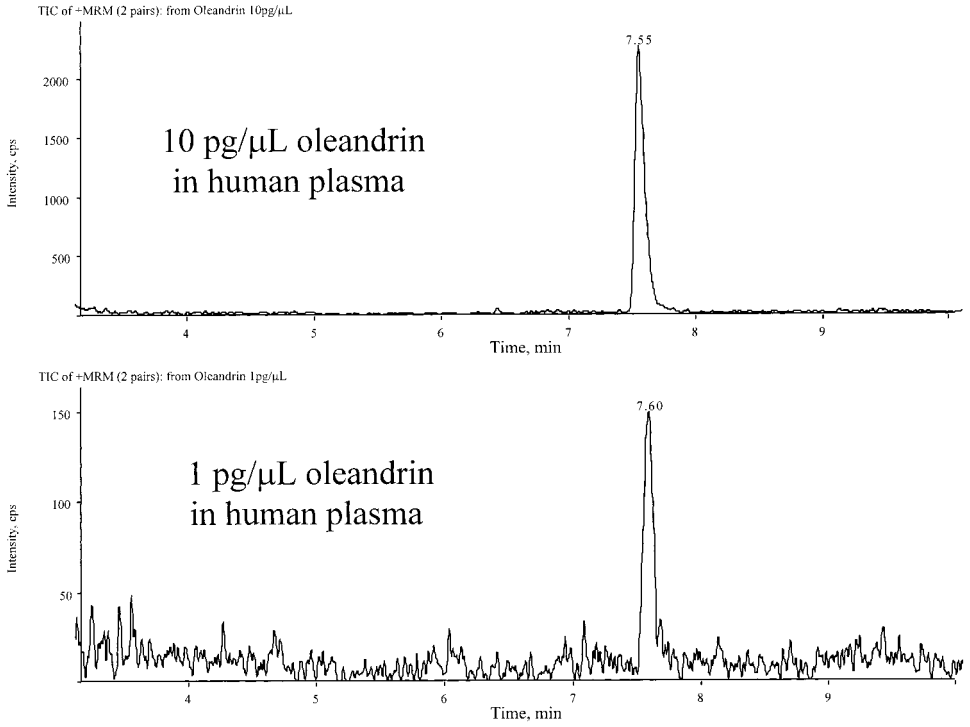

Figure 4. Multiple reaction monitoring (MRM) chromatograms of oleandrin in control human plasma in 10 pg/μL (Top) and 1 pg/μL (Bottom) for the precursor-to-product-ion transition of m/z 577 → m/z 373.

Matrix effects derived from human plasma were not deleterious with respect to t_r, as noted in the elution profiles of Figure 5. Only a 6-s change in t_r was noted for oleandrin between the human plasma control and a human volunteer's plasma that was sampled 30 min after an intramuscular dosing with 15 mg of oleander extract. Additional components in the m/z 577 → 373 transition from the volunteer's plasma may be attributed to the oleandrin isomers of cryptograndoside-A and nerigoside; t_r references for the presence of these components are provided in the middle trace, which profiles the m/z 577 → 373 transition from an oleander extract.

From the t_r confirmation of oleandrin in human plasma and the calibration curve generated, it was possible to generate a plasma concentration−time profile for a volunteer who was given

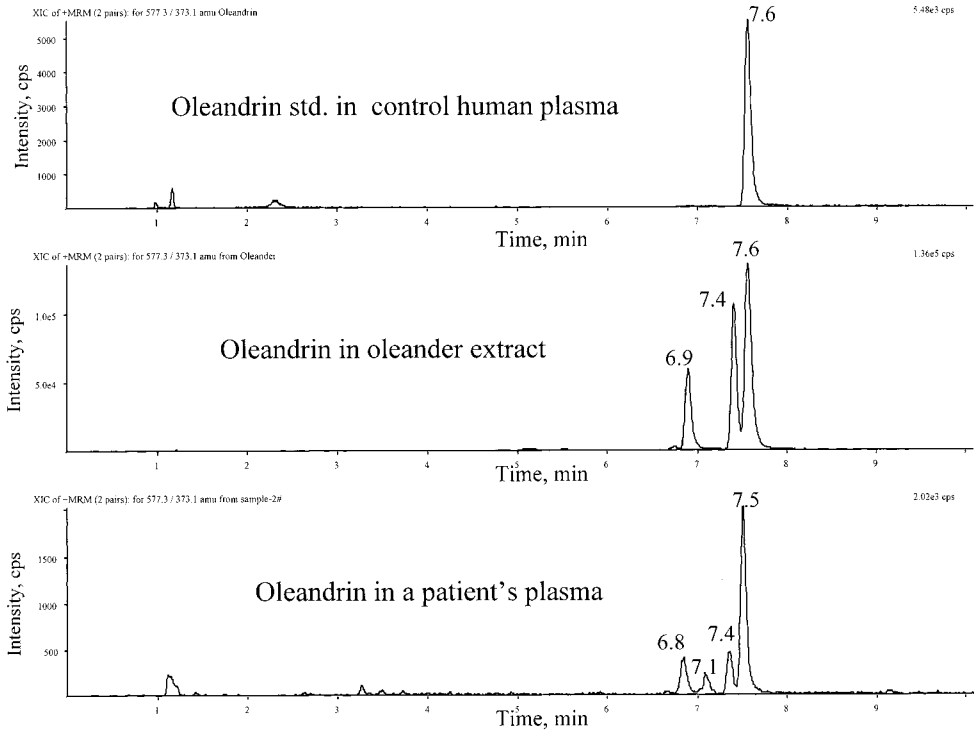

Figure 5. The MRM chromatograms of oleandrin in control human plasma (Top), in oleander extract (Middle), and in the volunteer's plasma (Bottom).

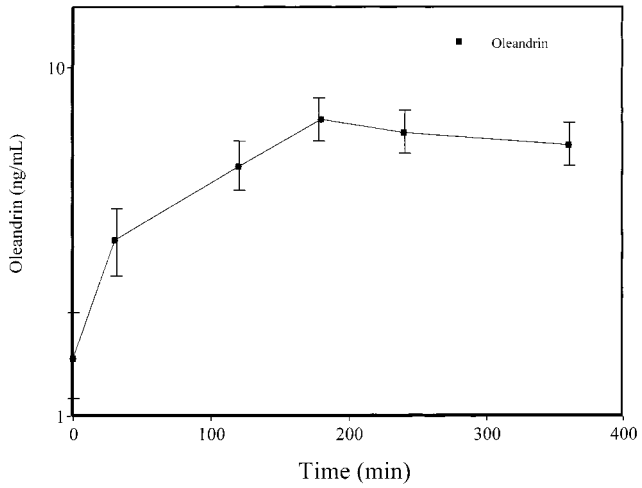

Figure 6. Plasma concentration−time profile of oleandrin obtained from a volunteer administered 15 mg of oleander extract. Each point of the concentration−time profile was based on triplet measurements of oleandrin in the plasma.

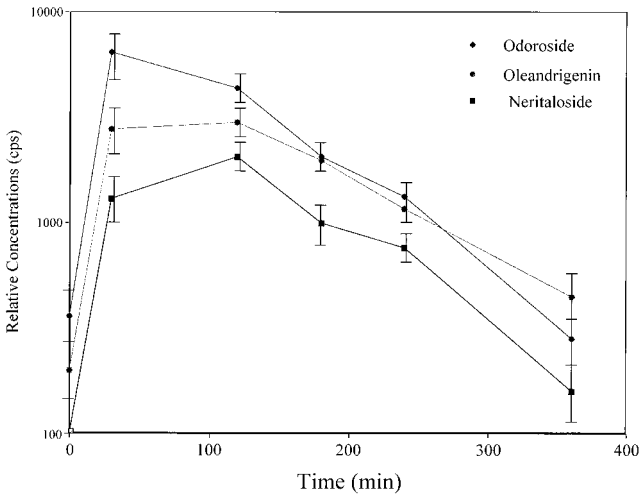

Figure 7. Relative concentration−time profiles of neritaloside, oleandrigenin, and odoroside obtained from a volunteer administered 15 mg of oleander extract. Each point of the concentration−time profiles was based on triplet measurements of odoroside, oleandrigenin, and neritaloside in the plasma, respectively.

an intramuscular dose of 15 mg of oleandrin contained within Anvirzel (Figure 6). From the profile, concentrations of oleandrin as high as 7 ng/mL were observed after 3 h. Furthermore, this concentration of oleandrin remained virtually asymptotic for the time trial of the study, thereby suggesting that there is a slow clearance of this cardiac glycoside from human plasma.

Although oleandrin is suspected to represent one of the principle biologically active components that is contained in oleander extract, we were interested in characterizing the concentration−time profiles of the other major components that were identified via LC/QqTOF (i.e., neritaloside, oleandrigenin, and odoroside). The MRM chromatograms of neritaloside, oleandrigenin, and odoroside in the volunteer's plasma after a 30-min dosing with 15 mg of oleander are profiled, although data in Figure 7 relate the relative response (in cps peak height) as a function of time for each component. Because neritaloside, oleandrigenin, and odoroside standards were not available commercially, the peak height was used to indicate the relative response for these three cardenolides.

CONCLUSION

The utility of accurate mass determinations and their application to herbal medicine research was illustrated by the identifica-

tion of neritaloside, oleandrigenin, odoroside, and oleandrin from a novel oleander extract. Clearly, accurate mass assignment at high resolution provides unique opportunities for the interpretation of CID spectra of the major components from herbal medicines.

The quantitative protocol for these target analytes, such as neritaloside, oleandrigenin, odoroside, and oleandrin, has been developed for application to pharmacokinetic studies. The oleandrin concentration in the oleander extract was 2.6 μg/mg of extract. The limit of quantitation for oleandrin is 20 pg; the dynamic range for oleander quantitation is broad, 1 ng/mL to 10 μg/mL. A linear dynamic range, spanning 4 orders of magnitude, was characterized by a correlation coefficient of 0.99. A plasma concentration−time profile for oleandrin from a volunteer who had been administered a therapeutic dose of Anvirzel (15 mg of oleander extract) was characterized to demonstrate the utility of this method. The relative concentrations of neritaloside, oleandrigenin, and odoroside in human plasma were also determined by this method.

LC/MS/MS technologies are extremely important for characterization and quantitation of herbal medicines because full characterization of these products is a desirable goal.[22,23] Analytical assays such as that described, in turn, can provide important pharmacokinetic and pharmacodynamic information that may be of relevance in helping to elucidate which components are the possible active ingredients within herbal medicines. With respect to Anvirzel, recent pharmacology studies have clearly shown that (1) oleandrin is a potent inhibitor of human tumor cell growth (IC50 3.0−10.0 ng/mL). Inhibition of human tumor cell growth is believed to be due to potent inhibition of Na, K-ATPase, leading to enhanced intracellular calcium concentration and activation of apoptotic pathways and (2) oleandrin-mediated inhibition of Na, K-ATPase also blocked cellular release of bFGF from human prostate PC3 and DU145 cells in a concentration- and time-dependent manner.[24]

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