

Murine pharmacokinetics and metabolism of oleandrin, a cytotoxic component of *Nerium oleander*

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(Received 4/24/2002; sponsored by Robert A. Newman 4/24/2002; accepted 4/25/2002)

Pharmacokinetic studies of [^3H]oleandrin, a cardiac glycoside component of Anvirzel[®], were conducted in mice after either an i.v. dose (40 $\mu\text{g}/\text{kg}$) or a p.o. dose (80 $\mu\text{g}/\text{kg}$). Oleandrin was rapidly absorbed after oral dosing (C_{max} at 20 min) although the elimination half-life was longer (2.3 ± 0.5 h) than that after i.v. dosing (0.4 ± 0.1 h). The $\text{AUC}_{0-\infty}$ values obtained after i.v. and p.o. dosing were 24.6 ± 11.1 and 14.4 ± 4.3 (ng-h/ml), respectively, resulting in an oral bioavailability of approximately 30%. After i.v. administration, oleandrin concentration in liver was approximately twice that measured in heart or kidney tissue. Oleandrogenin, the aglycone of oleandrin, was also found in these tissues. At 5 min, >60% of the total radioactivity in liver was due to oleandrin while 28% of the given dose was present as oleandrogenin. Twenty-four hours following injection, 8% of total radioactivity was excreted in urine and contained both oleandrogenin (4.4% of the injected dose) and oleandrin (1.9%). Sixty-six percent of injected radioactivity was found in feces and consisted of oleandrin and oleandrogenin in equal amounts. Uptake of oleandrin in brain after i.p. injection of oleandrin (3 mg/kg) or oleander extract (700 mg/kg) was examined. Measured by LC/MS/MS, oleandrin content in brain was higher following injection of extract than it was with an equivalent dose of oleandrin. The data suggest that components within oleander extract may enhance transport of oleandrin across the blood brain barrier.

Keywords: Anticancer drug, oleandrin, oleander extract, pharmacokinetics, mice.

INTRODUCTION

Nerium oleander is an ornamental plant widely distributed in subtropical Asia, south-

western United States, and the Mediterranean. Its medical and toxicological properties have long been recognized. It has been used, for example, in the treatment of hemorrhoids, ulcers, leprosy, snake bites, and even in the induction of abortion (1–3). Oleandrin (Figure 1), an important component of oleander extract (Anvirzel[®]) is a potent inhibitor of human tumor cell growth (4). Oleandrin-mediated cell death is associated with calcium influx, release of cytochrome C from mitochondria, proteolytic processes of caspases 8 and 3, poly (ADP-ribose) polymerase cleavage, and DNA fragmentation (5–8). Using LC/MS/MS, oleandrin, oleandrogenin (Figure 1) and two other metabolites were detected in a volunteer after an i.m. injection of oleander extract (9). Although a Phase I trial of Anvirzel[®] has recently been completed in patients with advanced solid tumor in the United States, only limited clinical pharmacokinetic information has been made available (10). We have, therefore, carried out pharmacokinetic and tissue distribution studies of oleandrin in mice after i.v., oral, and i.p. routes of administration.

MATERIALS AND METHODS

Chemicals

Oleandrin and oleandrogenin were obtained from Sigma (St. Louis, MO). Uniformly [^3H]labeled oleandrin (radiochemical purity, $\geq 98.9\%$, specific activity, 4 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). Oleander extract was obtained from Ozelle Pharmaceuticals (San Antonio,

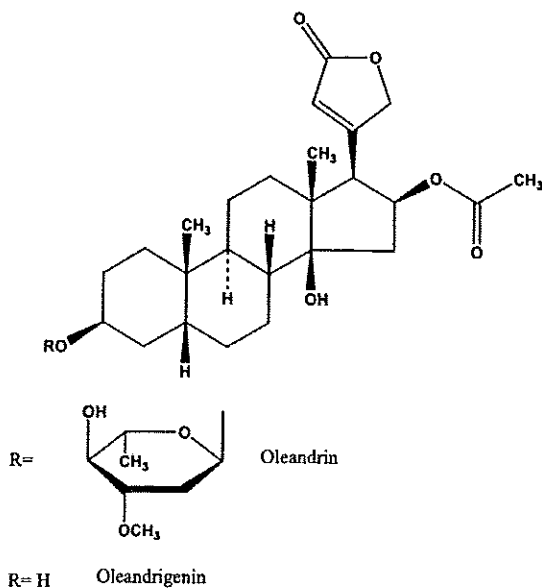


Figure 1. Oleandrin and oleandrigenin.

TX). Methoxyflurane was purchased from Schering-Plough Animal Health (Union, NJ). Methanol, acetonitrile, ethyl acetate, and formic acid were of HPLC grade and purchased from Fisher Scientific (Pittsburgh, PA). Universal scintillation cocktail was purchased from ICN Radiochemicals (Irvine, CA).

Instrumentation

The Waters HPLC apparatus consisted of a Model W600 pump, Model W715 automatic injector, and system controller. The HPLC was connected to a radioactivity detector (Model 500TR, Packard, Downers Grove, IL) for measuring oleandrin and oleandrigenin. A liquid scintillation counter (Beckman Model LS 6500, Fullerton, CA) was used to measure total radioactivity in mouse plasma and tissue samples. To increase assay sensitivity for mouse plasma and brain samples, LC/MS/MS analyses of oleandrin and oleandrigenin were also performed using a Quattro Ultima LC/MS/MS tandem mass spectrometer (Micromass, Beverly, MA). Waters tC18 cartridges (Sep-Pak[®], 50 mg) were used for solid-phase drug extraction.

Animals

Male CD-1 mice (20–25 g) were obtained from Harlan Laboratories (San Diego, CA). Water and food were available ad libitum. Animals

were housed in a standard controlled environment (temperature range, $70 \pm 2^\circ\text{F}$; relative humidity range, $55 \pm 2\%$). Five mice per group were used at each time point. Samples obtained from each mouse were processed separately. Results are expressed as mean \pm S.D. Animal studies were approved by UT-M. D. Anderson Cancer Center's Animal Care and Use Committee.

Metabolism of [³H]Oleandrin in Human and Mouse Plasma In Vitro

An aliquot (0.5 μCi , 72.1 ng) of [³H]oleandrin was added to 1 ml of human or mouse plasma. Samples were incubated at 37°C for 1, 3, and 6 h prior to addition of acetonitrile (2 ml). A "0" time control was prepared by adding 1 ml acetonitrile to plasma spiked with [³H]oleandrin immediately prior to immersing samples in ice without incubation. Samples were centrifuged at $10,000 \times g$ for 10 min and 50 μl of the supernatant was analyzed using HPLC with the radioactivity detector as described below.

Pharmacological Studies in Mice

Bioavailability [³H]oleandrin (40 μg with 280 μCi of [³H]oleandrin/kg) was administered i.v. to mice. For oral studies, mice were fasted overnight and a dose of 80 $\mu\text{g}/\text{kg}$ was administered. At designated times, mice were lightly anesthetized with methoxyflurane prior to decapitation. Blood samples were collected in tubes with heparin and centrifuged at $600 \times g$ for 10 min to obtain plasma. Plasma (0.2–0.5 ml) was diluted with 1 ml of milli-Q water and loaded onto a Sep-Pak tC18 cartridge, which was washed with 2 ml milli-Q water. The analytes were eluted with 2 ml ethyl acetate and evaporated to dryness under nitrogen. The residue was dissolved in 100 μl methanol and subsequently analyzed by HPLC with the radiometric detector. Liver, kidney, heart, and brain were also obtained from mice. Tissues were homogenized with four volumes (by weight) of saline. All procedures were carried out at 4°C . The homogenate (1–2.5 ml) was extracted twice with 3 ml acetonitrile and centrifuged at $1000 \times g$ for 5 min. The combined supernatants were evaporated to dryness under nitrogen and reconstituted with 1 ml milli-Q water. The

content of oleandrin and its major metabolite in tissues were analyzed as described for plasma.

Excretion

Mice were injected i.v. with [³H]oleandrin (40 µg with 280 µCi of [³H]oleandrin/kg) and housed in polycarbonate metabolism cages. Separate samples of urine and feces were collected at 0, 4, 7, 24, and 48 h. Urine was centrifuged at 10,500 × *g* for 10 min and supernatant was extracted and processed as described for plasma. Feces were homogenized with milli-Q water (1:3 w/v) and processed as described for tissue. To measure total radioactivity, an aliquot (100 µl) of homogenate was counted with 15 ml scintillation cocktail. An HPLC with a radiometric detector was used for analyzing the urine for unchanged drug and metabolites; all analyses were performed in triplicate.

Drug Distribution in Brain

In our preliminary studies, substantial amounts of oleandrin were found in brains of mice after i.v. injection of [³H]oleandrin. Further studies of drug content in brain tissue were conducted in mice injected i.p. with oleandrin (3 mg/kg) or oleander extract (700 mg/kg, an equivalent dose of oleandrin). Plasma and brain tissue were collected at 0.5, 1, 2, 8, and 24 h and processed as described above. Drug content was determined using the LC/MS/MS method as described below.

HPLC Assay of [³H]Oleandrin and Its Metabolites

Separation of oleandrin and its metabolites was achieved by HPLC through a reverse-phase analytical column (Phenomenex Spherisorb 5 ODS, 250 × 4.5mm) preceded by a µBondapak C18 guard column (Waters, Milford, MA). A mobile phase of acetonitrile and water (47:53, v/v) was used at a flow rate of 0.9 ml/min. Drug and related metabolites were detected using both UV and radiometric detectors as described above.

LC/MS/MS Assay of Oleandrin and Oleandrogenin

The LC/MS/MS method for determination of oleandrin and oleandrogenin was performed essentially as described by Wang et al. (9).

Briefly, LC separations were achieved using a YMC ODS Aqua 3-µm analytical column (2 × 100 mm I.D.) protected by a YMC ODS Aqua 5-µm (2 × 10 mm) guard column. A linear gradient mobile phase was used by mixing 0.05% formic acid (pH 3.0) and methanol (20–95%, v/v) over 5 min at a flow rate of 0.3 ml/min. The analytical column was kept at 40°C. Oleandrin and oleandrogenin were identified in the oleander extract by retention time, mass weight, and daughter ion spectra compared to commercially available reference standards. Concentrations of oleandrin and oleandrogenin were determined by quantifying the peak area for the primary product ion of each substance, *m/z* 577.3 > 373.4 for oleandrin and *m/z* 434.4 > 373.7 for oleandrogenin. Cone voltage for both analytes was 55 V with collision energy settings at 15 and 12 V for oleandrin and oleandrogenin, respectively.

Pharmacokinetic Analyses

Pharmacokinetic parameters for the single i.v. and p.o. dosing of [³H]oleandrin were estimated by fitting a two-compartment open linear model, using weighted nonlinear least-squares analysis to the plasma concentration-time data obtained from five animals. All of the pharmacokinetic parameters (*t*_{1/2}, *k*, *T*_{max}, *C*_{max}, AUC_{0-∞}, Cl_T, and *V*_{es}) were estimated by WinNonlin 3.1 (Pharsight, Mountain View, CA).

Statistic Analyses

A Student *t* test was performed to compare drug content in brain tissue of mice after oleandrin or oleander extract treatment. Differences between mean drug content were considered to be statistically significant at *P* < 0.05.

RESULTS

Metabolism of [³H]Oleandrin in Mouse and Human Plasma in Vitro

As shown in Figure 2, oleandrin was converted to oleandrogenin in mouse plasma in a time-dependent manner. Two additional unknown radiolabeled peaks eluting at 2.9 and 4.0 min were observed. These peaks comprised approximately 20% of the total recovered radioactivity following the 6-h

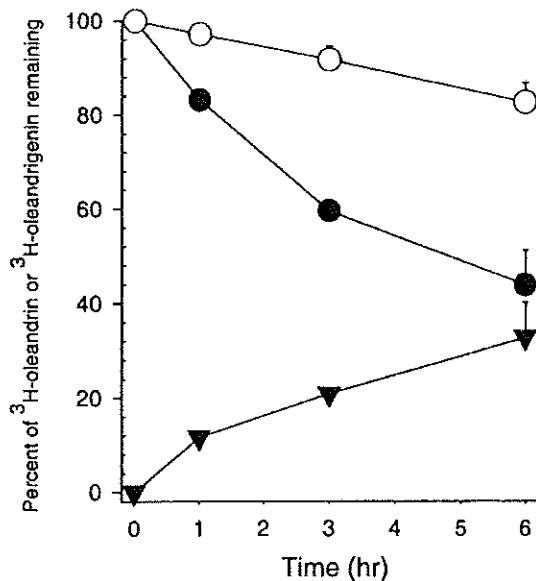


Figure 2. In vitro metabolism of [³H]oleandrin in mouse and human plasma at 37°C. [³H]oleandrin (●) was converted to [³H]oleandrigenin (▼) in mouse plasma but not in human plasma. Open circles designate oleandrin (○) content in human plasma. Results are presented as mean ± SD (*n* = 3).

incubation. When human plasma was incubated with oleandrin up to 6 h, however, no oleandrigenin was formed. Only one minor peak accounting for 13% of total radioactivity was observed at 2.9 min.

Plasma [³H]Oleandrin in Mice After I.V. and P.O. Administration

[³H]oleandrin concentrations in plasma after i.v. or p.o. administration of [³H]oleandrin are presented in Figure 3A. After p.o. administration, [³H]oleandrin was rapidly absorbed with a maximum concentration (*C*_{max}) of 7.2 ng/ml achieved at about 20 min (*T*_{max}). This *C*_{max} after p.o. administration, however, was 1% of that following i.v. injection. The [³H]oleandrin concentrations in plasma after p.o. administration decreased slowly with a longer half-life than that observed after intravenous injection. The oral bioavailability (*F*) was approximately 30% and was calculated according to the following formula:

$$F(\%) = \frac{AUC_{0-\infty, p.o.} \cdot Dose_{i.v.}}{AUC_{0-\infty, i.v.} \cdot Dose_{p.o.}} \times 100$$

Pharmacokinetic parameters derived following i.v. and p.o. administration of oleandrin are summarized in Table 1.

Tissue Distribution of [³H]Oleandrin

Distribution of oleandrin in mouse kidney, liver, and heart tissue after i.v. injection of [³H]oleandrin is shown in Figure 3B. Peak levels of [³H]oleandrin occurred in these tissues within 5–15 min following injection. The highest concentration found in liver was almost twice as high as that in heart or kidney tissue. Conversion of oleandrin to its aglycone metabolite was rapid; within 5 min, approximately 27% of the administered dose of oleandrin in liver were oleandrigenin. Conversion of oleandrin to oleandrigenin in liver tissue and plasma is shown in Figure 4. In addition to oleandrigenin, 10% of the radioactivity in mouse liver was associated with an unidentified metabolite peak eluted at 5.6 min on the chromatographic analysis. In contrast, oleandrigenin was the only detected metabolite present in kidney and heart tissue (data not shown). The concentration of oleandrigenin increased while oleandrin levels decreased resulting in smaller ratios of oleandrin to oleandrigenin over time. The ratios decreased in heart from 13.5 to 4.5, 3.5, 1.0, and 0.6 at 5, 15, 30, 60, and 120 min, respectively. The ratios similarly decreased in kidney from 10.1 to 4.2, 2.6, 1.0, and 0.45 over time. By 4 h, only oleandrigenin was detected in both heart and kidney tissue.

Oleandrin Persists in Brain

Preliminary studies showed that substantial amounts of oleandrin could be found in mouse brain following either i.v. injection or p.o. administration of [³H]oleandrin. To further explore this finding, mice were injected i.p. with either non-radiolabeled oleandrin (3 mg/kg) or oleander extract (700 mg/kg, equal to an equivalent dose of oleandrin). A sensitive LC/MS/MS method was used to quantify drug in brain tissue. Brain and plasma were sampled at various times and analyzed for oleandrin content. Relatively high concentrations of oleandrin were found in brain tissue and remained elevated even 8 and 24 h after a single injection of drug (Table 2). Of interest was the observation that no metabolite, including oleandrigenin, could be detected in brain tissue. Additionally, while plasma concentrations of oleandrin were considerably higher immediately after administration of either

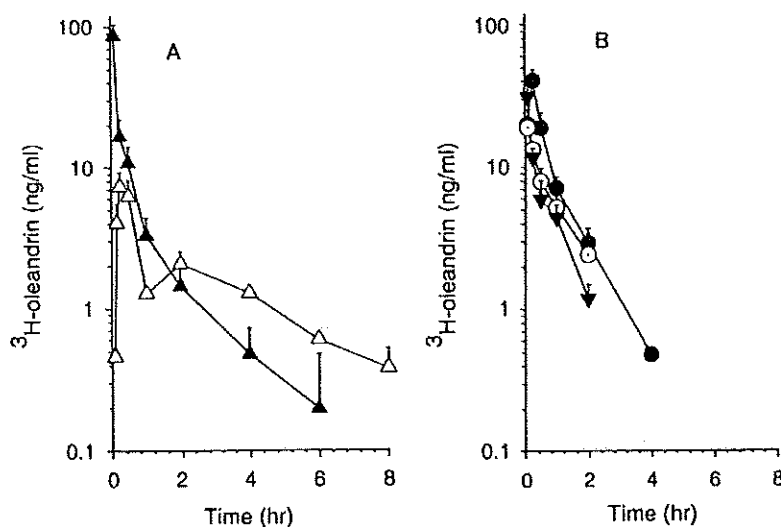


Figure 3. (A) Comparison of plasma concentrations of [³H]oleandrin in mice after i.v. (▲) and oral (△) administration of [³H]oleandrin. (B) [³H]oleandrin concentrations in mouse kidney (○), liver (●), and heart (▼) after i.v. administration of [³H]oleandrin. Data are presented as mean ± SD (*n* = 5).

oleandrin or oleander extract, brain tissue concentration rose rapidly and remained higher than plasma concentration for a protracted period of time. Table 2 also illustrates the increasingly higher ratios of oleandrin content in brain relative to that in plasma over time. Although oleander extract injected at an equivalent dose of oleandrin, ratios of oleandrin in brain relative to that in plasma were significantly higher in mice after oleander extract injection than those mice after oleandrin injection.

Cumulative Excretion

Twenty-four hours after mice received an i.v. dose of [³H]oleandrin (40 μg/kg), 60% were recovered in feces, and less than 10% of the total radioactivity were excreted in urine (Figure 5). When samples were collected for an additional 24 h, only 6% more radioactivity was recovered in feces and only 1% more of the injected dose recovered from urine. Feces

contained an approximately equal amount of oleandrin (16.5%) and oleandrogenin (17.3%). The remaining radioactivity was due to two additional unidentified metabolites. In contrast, urine contained more oleandrogenin (4.4%) than oleandrin (1.9%) and only one unknown metabolite eluting at 5.8 min on the chromatogram was found.

DISCUSSION

When incubated with mouse plasma, oleandrin was converted to its aglycone metabolite oleandrogenin (Figure 1). Incubation with human plasma, however, did not result in the formation of this aglycone even after a 6-h incubation. The presence of oleandrogenin in the plasma of a volunteer (9), however, suggests that oleandrogenin may be formed in tissues other than plasma. This indicates a species-dependent difference in

Table 1: Summary of pharmacokinetic parameters of oleandrin in mice

Route of administration	Dose (μg/kg)	<i>T</i> _{max} (h)	<i>C</i> _{max} (ng/ml)	<i>T</i> _{1/2α} (h)	<i>T</i> _{1/2β} (h)	AUC _{0-∞} (ng·h/ml)	Cl _T (l/h·kg)	<i>V</i> _{ss} (l/kg)	Bioavailability (%)
i.v.	40	–	791 ± 502	0.1 ± 0.02	0.4 ± 0.1	24.6 ± 11.1	1.13 ± 0.41	0.3 ± 0.2	
p.o.	80	0.3 ± 0.1	7.2 ± 3.5	0.4 ± 0.3	2.3 ± 0.5	14.4 ± 4.3	5.60 ± 1.71	2.6 ± 1.1	29.3

Values presented are mean ± S.D. of 5 animals.

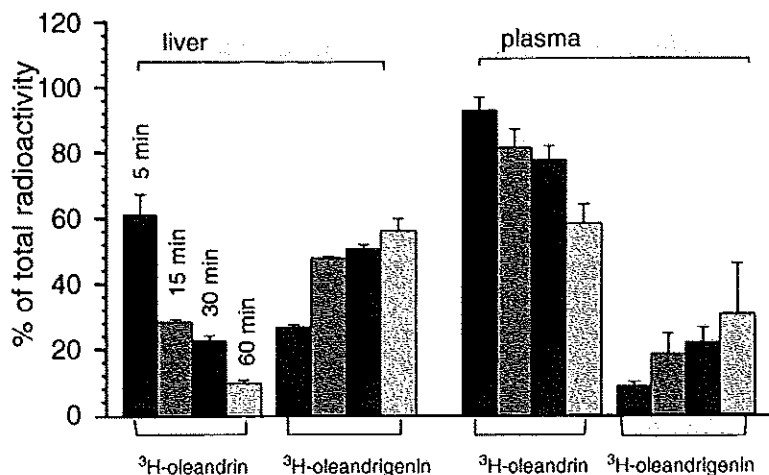


Figure 4. [³H]oleandrin concentrations in liver and plasma decrease in a time-dependent (5–60 min) manner while levels of [³H]oleandrigenin increase. Data are presented as mean \pm SD ($n = 5$).

oleandrin metabolism and also suggests that caution must be taken in processing mouse blood in order to prevent any ex vivo metabolism. Of interest was the fact that boiled mouse plasma lost its metabolic capability suggesting that conversion of oleandrin to oleandrigenin in this matrix may be an enzymatic process.

The initial disappearance of oleandrin from plasma was rapid with a short $t_{1/2}$ in mice following either an i.v. or p.o. dose of this cardiac glycoside. However, the $t_{1/2\beta}$ was longer in mice after the p.o. dose than that of i.v. dose. Cardiac tissue contained a considerable amount of oleandrin. This may partially explain the usefulness of oleandrin as a cardiac medicine as well as its potential for cardiac toxicity (1,2,11). The kidneys contained a rather low amount of oleandrin.

This is not too surprising as urinary excretion (<10% of the dose) was not a major route of elimination. A larger amount of oleandrin was found in liver tissue. By 24 h, 60% of the dose were excreted as equal amounts of oleandrin and oleandrigenin through feces suggesting hepatobiliary excretion. This finding is similar to that reported for other cardiac glycosides. For example ouabain, digoxin, and digitoxin have also been reported as being excreted in bile in high concentrations (12,13).

Oleandrin rapidly accumulated in mouse brain tissue, appearing as early as 30 min after i.p. injection, and remained an extended period of time. This indicates that oleandrin rapidly crosses the blood-brain barrier. While plasma oleandrin concentrations were initially much higher than those in brain they

Table 2: Relative concentrations of oleandrin in mouse brain and plasma after i.p. injection of oleandrin or oleander extract

Time (h)	Oleandrin concentration					
	Oleandrin (3 mg/kg)			Oleander extract (700 mg/kg) equivalent dose of oleandrin		
	Brain (ng/g)	Plasma (ng/ml)	Ratio of brain to plasma	Brain (ng/g)	Plasma (ng/ml)	Ratio of brain to plasma
0.5	85 \pm 43	442 \pm 32	0.2 \pm 0.1	451 \pm 127	887 \pm 409	0.5 \pm 0.1*
1	99 \pm 30	502 \pm 18	0.2 \pm 0.1	642 \pm 91	904 \pm 176	0.7 \pm 0.1**
2	103 \pm 41	405 \pm 96	0.3 \pm 0.2	554 \pm 111	801 \pm 206	0.7 \pm 0.1**
8	239 \pm 88	90 \pm 37	2.7 \pm 0.8	537 \pm 36	45 \pm 25	11.9 \pm 2.7**
24	130 \pm 58	18 \pm 4	7.2 \pm 1.9	550 \pm 44	2 \pm 1	275 \pm 95**

The ratio of oleandrin in brain to plasma after injection of oleander extract is significantly greater than that of oleandrin injection (* $P < 0.05$ and ** $P < 0.01$).

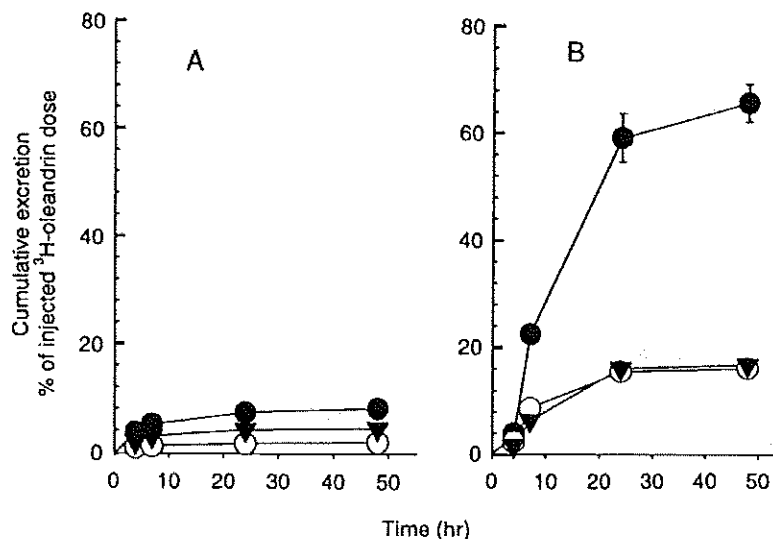


Figure 5. Cumulative excretion of total [³H]radioactivity (●), [³H]oleandrin (○) and [³H]oleandrigenin (▼) in urine (A) and feces (B) of mice after i.v. injection of [³H]oleandrin. Data are presented as mean ± SD (*n* = 5).

subsequently decayed rapidly. This resulted in higher ratios of brain to plasma content of oleandrin over time. The persistence of high oleandrin concentrations in mouse brain may explain the previously reported cardiac glycoside mediated toxicity in the central nervous system (3,14,15). A methanolic extract of *N. oleander* leaves containing four cardenolides (neritaloside, nerizoside, neridinose, and odoroside-H) has been shown to produce central nervous system depression in mice (14,15). In spite of this potential for CNS toxicity, the penetration of oleandrin into brain tissue suggests it may be useful in the treatment of brain tumors. Our laboratory has recently shown that oleandrin inhibited the growth of glioblastoma cells in vitro (R. A. Newman et al., personal communication). Furthermore, mice receiving oleander extract were found to have even higher concentrations of oleandrin in brain and plasma than mice receiving an equivalent dose of oleandrin. This suggests that some other components of the oleander extract may enhance oleandrin's ability to cross the blood brain barrier. A phase I clinical trial of oleander extract (Anvirzel[®]) demonstrated that i.m. administration at doses up to 1.2 ml/m²/day (or 18 mg/m²/day) are safe. The most commonly observed toxicity was mild pain at the injection site and myalgia, but no serious dose-limiting toxicities were found

(10). These data suggest that oleander extract may have an application in the treatment of other CNS malignancies.

Using LC/MS/MS analyses, oleandrin, oleandrigenin, and neritaloside were identified in a sample of oleander extract and from the plasma of a volunteer who received an i.m. injection (15 mg) of oleander extract (9). Oleandrin reached a maximum plasma concentration in 3 h (9). Our study showed that the *C*_{max} was reached in less than 0.5 h in mice after an i.p. dose of oleander extract. At the present time, observed differences in absorption rate, which may be related to the species used and/or the routes of administration can not be excluded. Further studies comparing pharmacokinetics of oleandrin after various routes of administration, in multiple species, are clearly needed.

ACKNOWLEDGEMENTS

This work was made possible in part by a grant from Anviram, Inc. (San Antonio, TX). The authors also thank Ms. Norma Ramirez for skillful typing and manuscript processing.

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