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Tissue Distribution of Doxorubicin and Doxorubicinol in Rats Receiving Multiple Doses of Doxorubicin

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Summary. *Plasma and tissue levels of doxorubicin (DXR) and doxorubicinol (DXR-OL) were measured fluorometrically after high-pressure liquid chromatography at 1, 3, and 24 h following one, nine, and 24 doses of 1.0 mg DXR/kg or one and eight doses of 4.0 mg DXR/kg, IP, to rats. Comparison of plasma levels of DXR found following single and multiple doses suggests significant build-up of DXR at 1 h with successive doses, but not at 3 h. Liver exhibited substantially higher levels of DXR (on a per gram of protein basis) than did plasma, and multiple doses did not produce higher levels than did a single dose. In contrast, the heart accumulated DXR slowly, attaining levels after multiple dosing in excess of those found in the liver. Skeletal muscle exhibited dose-related levels similar to those for heart but the absolute levels of DXR in muscle were only about one-tenth of those observed in heart. DXR-OL was at very low levels of ≤ 4% of the DXR levels in the tissues; it was, however, a major circulatory metabolite, attaining levels in the plasma as high as 85% of the concentration of DXR.*

Introduction

The clinical use of doxorubicin (DXR) is restricted by dose-dependent cardiomyopathy [4, 5] and the development of a rat model system for prediction of this effect in man has been reported [20]. In this model system daily IP injections of DXR or analogs are employed. As part of a program to evaluate this rat model system for assessing the comparative cardiotoxic potential of DXR and its analogs [9], we determined the levels of DXR and its major cytotoxic metabolite, doxorubicinol (DXR-OL) [11], in plasma and selected tissues of rats receiving single and multiple doses of DXR. This study differs from earlier studies in rats in which DXR and metabolites were measured after only single doses [16] or no measurements of drugs were performed in rats receiving multiple doses of DXR [10].

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Materials and Methods

DXR was supplied by the National Cancer Institute and by Adria Laboratories, Inc. A synthetic reference sample of DXR-OL, as a mixture of the C-13 diastereoisomers, was prepared by reduction of DXR with sodium cyanoborohydride [15]. Solutions for injection were prepared by dissolving DXR in polyethylene glycol 200 : isotonic saline (2 : 1 by volume) to obtain concentrations of 1 or 4 mg DXR/ml. The rats received IP injections of 1.0 ml/kg to obtain the doses of 1.0 and 4.0 mg/kg.

Female Sprague-Dawley rats (Simonsen Laboratories, Inc.; Gilroy, CA, USA) weighing 170–200 g were used. They were maintained on food and water ad libitum. In the first experiment, 18 rats received a dose of 1.0 mg/kg between 8:00 and 9:00 a.m. Six rats received corresponding doses of the vehicle only. Following one treatment with DXR, we sacrificed two rats each at 1, at 3, and at 24 h. Heparinized plasma, heart, liver, and skeletal muscle were collected for drug analysis. The remaining 12 experimental rats received the same dose of DXR daily for the next 4 days, were rested for 2 days, and then again received doses of 1.0 mg DXR/kg for the next 4 days. At this time, we again sacrificed two rats at 1, at 3, and at 24 h, obtaining plasma and tissues for analysis as before. The remaining six rats continued to receive 1.0 mg DXR/kg 5 days per week until they had received 24 doses, at which time they were sacrificed on the same schedule as the others. Control rats received injections of the vehicle 5 days per week throughout. The mean initial body weight of the control group was 190 ± 3.6 g (standard error); that of the experimental group was 176 ± 3.2 g. The body weights of the control group and the experimental animals were determined twice per week throughout the experiment.

Subsequently, a similar experiment was initiated in ten rats receiving doses of 4 mg DXR/kg/day by the same route in the same vehicle. In this study, two rats were sacrificed at 1 h and single rats at 3 and 24 h after the first dose. One rat died after receiving six doses and two died after seven doses of 4 mg DXR/kg. The three remaining rats were given an eighth dose and one was sacrificed at 1 h and two at 24 h post-treatment. Blood and tissues from all sacrificed animals were obtained as in the experiment employing

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the lower dose. The initial mean body weight of a control group of six rats was 213 ± 7.0 g, as against a mean body weight of 213 ± 2.4 g for this experimental group. All plasma and tissue samples were stored frozen until analyzed.

For the detection and quantitation of DXR and DXR-OL, we chromatographed extracts of plasma or tissues at ambient temperature (22° C) on a 5-μm Lichrosorb SI-60 column (10 × 250 mm, Altex Scientific Co., Berkeley, CA, USA), using a mobile phase of chloroform : methanol : acetic acid: 4.5 m*M* $MgCl_2$ (37 : 10 : 2 : 1; v/v). Fluorescence detection and quantitation of the resolved compounds in the effluent liquid were accomplished with the aid of the equipment and characteristics previously described [12]. Control plasma and tissue samples spiked with DXR or DXR-OL served as standards for quantitation of unknowns by peak heights. All glassware used to extract DXR and DXR-OL from plasma ot tissue homogenates was silylated [8].

To extract DXR and DXR-OL from plasma, we shook a mixture of 1.0 ml plasma, 0.25 ml 1.0 *M* phosphate buffer, pH 8.0, and 10 ml chloroform : methanol (4 : 1, v/v) for 20 min at 4° C. After centrifuging to separate the phases, 7.0 ml of the organic phase was evaporated to dryness at 20° C under vacuum in a vortex-evaporator. The residue was dissolved in 700 μl HPLC mobile phase and 500 μl was chromatographed. A mean recovery of 93.4% of added DXR to control plasma (4.5–236 ng/ml) was obtained (coefficient of variation was 10.5%). Similar amounts of DXR-OL added to control plasma yielded a mean recovery of 97.8% (CV = 13.3%).

Liver, heart, and muscle samples were homogenized in a mixture of 1.5 ml of 0.2 m*M* $MgCl_2$ in 0.07 *M* borate buffer, pH 8.5, and 0.1 ml oxalic acid solution (7.5 mg/ml). One milliliter of the homogenate was mixed with 1.0 ml saturated $(NH_4)_2SO_4$ in 0.4 *M* borate buffer, pH 8.5, and shaken gently at 4° C for 15 min. The samples were then extracted with 10 ml chloroform–methanol (4 : 1, v/v) for 20 min at 4° C. After centrifugation, the aqueous phase was aspirated off and the organic phase was decanted into a clean tube and washed for 5 min by shaking with 1.0 ml of the above $MgCl_2$-borate buffer. After separation, the washed organic phase (7.0 ml) was evaporated to dryness, the residue dissolved in mobile phase, and an aliquot chromatographed as described above. The mean recoveries from 24–246 ng DXR added to liver, heart, and muscle from control animals were 88.7% (CV = 6.7%), 96.8% (CV = 14.2%), and 90.6% (CV = 5.1%), respectively. Mean recoveries of the same amounts of DXR-OL were 73.4% (CV = 5.0%), 70.0% (CV = 11.7%), and 64.7% (CV = 8.9%), respectively.

Because the extent of hydration of various tissues in various animals may contribute to differences in concentrations of DXR or DXR-OL per gram of wet weight of tissue, we determined the concentrations of protein in all tissues by a dye-binding procedure, using human serum albumin (fraction V) as the reference protein [1]. Subsequently, we expressed the levels of DXR and DXR-OL as micrograms per gram of protein.

Results and Discussion

There was a significant ($P < 0.05$) decrease of 23% in the mean body weight of the rats receiving a total of 24 doses of 1.0 mg DXR/kg compared with the vehicle-treated controls. In the study in which 4.0 mg DXR/kg/day was given, we also observed a significantly lower mean body weight of 23% in the group receiving six doses of DXR compared with their control group. These observations suggest a dose-dependent cumulative overall toxicity similar to that noted earlier by others [10] who employed thrice-weekly doses of 1 mg DXR/kg or once-weekly doses of 1 or 2 mg DXR/kg and found significant decreases in body weight only in rats receiving three or more doses of 2 mg/kg.

Table 1 presents the mean values of DXR and DXR-OL we found in the plasma and tissues of the

Table 1. Mean tissue levels (μg/g protein) of DXR and DXR-OL in rats receiving 1.0 or 4.0 mg DXR/kg IP

Dose and number of treatments	Time after last treatment (h)	Tissue							
		Plasma		Liver		Heart		Muscle	
		DXR	DXR-OL	DXR	DXR-OL	DXR	DXR-OL	DXR	DXR-OL
1.0 × 1	1	0.086	0.014	8.2	0.09	4.3	< 0.3	0.38	< 0.07
	3	0.11	0.036	17	0.26	4.9	< 0.3	0.40	< 0.07
	24	0.019	0.007	26	0.14	7.4	1.6	0.58	< 0.07
1.0 × 9	1	0.23	0.077	22	0.53	40	1.6	2.2	0.13
	3	0.092	0.038	20	0.36	28	1.3	3.6	0.18
	24	0.034	0.029	8.6	0.24	21	1.8	2.4	0.24
1.0 × 24	1	0.34	0.050	20	0.46	38	1.7	5.0	0.28
	3	0.053	0.021	10	0.34	39	2.0	4.2	0.24
	24	0.066	0.026	6.4	0.28	27	1.8	5.3	0.42
4.0 × 1	1	0.88	0.10	40	0.19	30	0.22	5.8	< 0.12
	3[a]	0.25	0.09	25	5.0	38	0.67	12	0.21
	24[a]	0.10	0.02	11	0.43	7.8	0.63	9.2	0.52
4.0 × 8	1[a]	2.6	0.67	75	1.4	170	13	24	2.8
	24	1.4	0.12	27	1.2	194	15	38	4.5

[a] At these times, values are from single observations

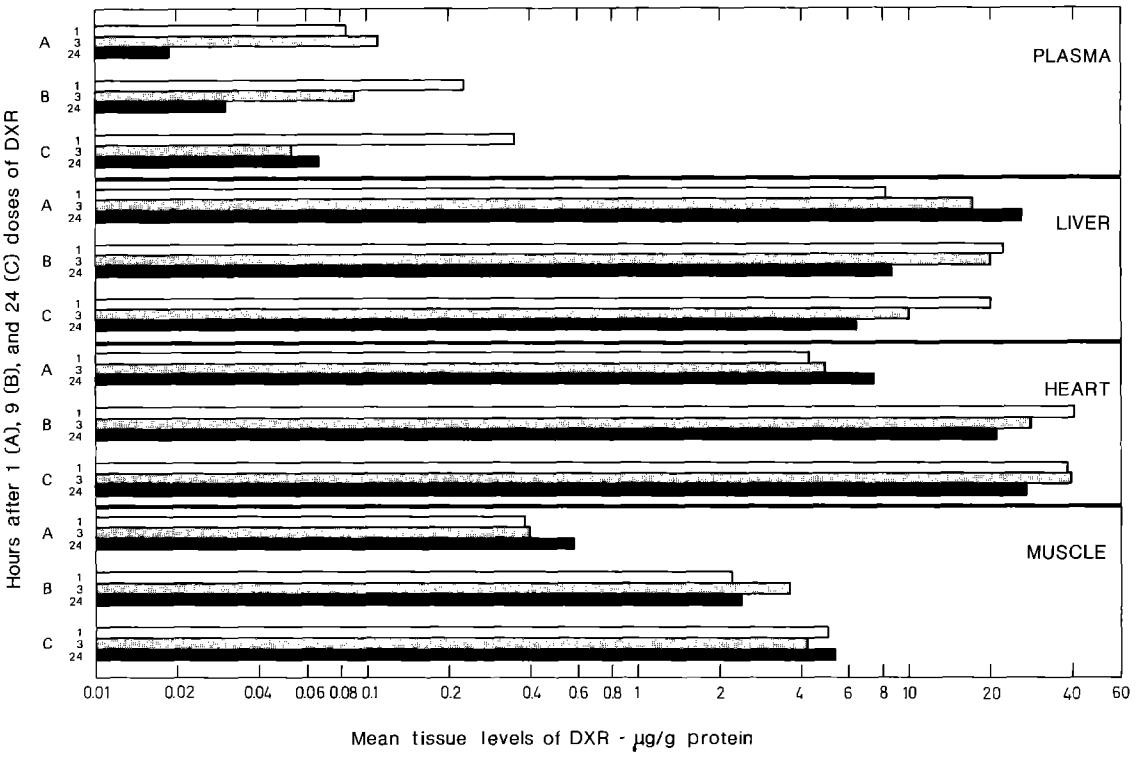

Fig. 1. Mean levels of DXR (µg/g of protein) in plasma and tissues of rats at 1, 3, and 24 h following one, nine, and 24 doses of 1.0 mg DXR/kg

rats receiving 1.0 or 4.0 mg DXR/kg. From the data of column 3 it is apparent that plasma levels of DXR increased substantially at 1 h from the 1st to the 9th and 24th treatments of 1.0 mg DXR/kg. A similar pattern of increases in levels of DXR with increasing number of doses was observed at 24 h. In contrast, the 3-h plasma levels of DXR were progressively lower from the 1st through the 9th and 24th treatments. These changes, compared also in the upper panel of Fig. 1, suggest very complex relationships between absorption from the peritoneal cavity and clearance from the circulation that these experiments cannot address. Nevertheless, the progressive increase of DXR in the 24-h plasma samples suggests a relatively slow accumulation of DXR with this dosage schedule.

The plasma levels of DXR-OL (column 4 of Table 1) after one dose of DXR ranged from 16–37% of the DXR levels. After nine doses, DXR-OL was present in relatively larger amounts ranging from 33–85% of theDXR levels, and after 24 doses DXR-OL was still present in amounts ranging from 15–40% of the DXR levels. Clearly, this metabolite makes a substantial contribution to the total levels of drugs in the circulation following multiple DXR treatments. The levels of DXR and DXR-OL found in plasma after one and eight doses of 4.0 mg DXR/kg (Table 1) strongly support the accumulation with time of both DXR and DXR-OL observed after the lower doses of 1.0 mg/kg. Because DXR-OL exhibits significant activity in various test systems for anticancer action [11], such plasma levels of DXR-OL as we have found in these experiments suggest that the overall effects of administered DXR can be partly attributed to DXR-OL.

Rapid penetration into and substantial uptake of DXR by the liver are clearly indicated by the approximately 100-fold higher levels in this organ than in the plasma after only one dose of 1.0 mg DXR/kg (Table 1, Fig. 1). Also, whereas plasma levels declined at 24 h, those in liver were highest at this time after a single dose. However, after either nine or 24 doses we found the liver levels decreasing with time even though the highest level (22 µg/g at 1 h after nine doses) was nearly the same as the maximum level noted after a single dose (26 µg/g at 24 h). These results suggest that the liver attained an equilibrium concentration of DXR by 24 h after a single dose that was not increased following continued dosing for up to the total of 24 doses studied. The very limited observations after one and eight doses of 4.0 mg DXR/kg (Table 1), on the other hand, suggest accumulation after multiple doses in this organ. From the low levels of DXR-OL of only approximately 4%

of the DXR after either the low or high doses of DXR administered, we conclude that DXR-OL is not an important contributor to the total drug in the liver.

In contrast to the liver, the levels of DXR in the heart (column 7, Table 1, and Fig. 1) increased substantially following nine treatments with 1.0 mg DXR/kg compared with those observed after only one treatment. No substantial further increases were noted from 9–24 treatments. Thus it appears that unlike the liver, the heart accumulated DXR slowly. In addition, following multiple doses of either 1 or 4 mg DXR/kg, levels in heart tissue routinely were higher than levels in liver. Selective build-up of DXR in the heart of rats in this study appears to parallel earlier studies by others in mice [13, 14], in which selective accumulation or high retention in the heart was reported.

DXR-OL levels found in heart tissue were, as in the liver, at very low levels even after doses of 4.0 mg DXR/kg. These observations are similar to those of Blanchard et al. [3] that DXR-OL accounted for $< 1\%$ of the DXR found in the heart 30 min after the infusion of single doses of 20 mg DXR/kg to rats. They reported a level of DXR in the heart tissue of 410 μg/g protein, but did not study other times, other tissues, or multiple doses.

Columns 9 and 10 of Table 1 show that skeletal muscle exhibited levels of DXR and DXR-OL that were approximately one-tenth of those found in the cardiac muscle. Nevertheless, a pattern of delayed uptake typical of the heart (Fig. 1) was also noted in the muscle. Thus, it appears that the two muscle tissues accumulate DXR more slowly than liver.

The findings of this investigation suggest that single-dose experiments do not yield a true representation of levels of DXR in tissues in rat studies attempting to relate levels with pharmacologic effects typical of certain tissues such as cardiotoxicity. For evaluating the usefulness of this rat model system [20] for the prediction of potential cardiotoxicity of anthracyclines in man, we believe these limited observations on drug distribution in plasma and tissues emphasize the need for a combined multidisciplinary effort linking pharmacologic and drug measurements. The need for such linkage is clearly demonstrated by the reports that congestive heart failure in patients receiving DXR can be reduced dramatically by switching from regimens of 60–75 mg/m^2 once every 3 weeks to schedules of 17–20 mg/m^2 once weekly [7, 17–19]. Others have shown that a weekly schedule of lower doses results in greatly reduced peak plasma levels of drug compared with the every-3-weeks schedules [6]. Thus, it would be anticipated that DXR levels in human heart would also be dramatically reduced, perhaps below cardiotoxic levels. More recently, Benjamin et al. [2] have extended this concept by employing prolonged continuous IV infusions of DXR over up to 96 h. They concluded that the decreased peak plasma levels of DXR obtained with longer infusion times reduced cardiac toxicity. In neither this nor the earlier studies in which weekly dosage schedules were used was the antitumor activity of DXR compromised.

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