

## Disposition of $^{14}\text{C}$ -labelled 4'-epidoxorubicin and doxorubicin in the rat

### A comparative study

Federico Arcamone, Marco Lazzati, Gian Piero Vicario, and Guido Zini

Farmitalia Carlo Erba SpA – Ricerca & Sviluppo Chimico, Via Giovanni XXIII, 23, I-20014 Nerviano, Milano, Italy

**Summary.** The disposition of 4'-epi-[14- $^{14}\text{C}$ ]doxorubicinHCl (4'-epi-[ $^{14}\text{C}$ ]DXR) and [14- $^{14}\text{C}$ ]doxorubicinHCl ([ $^{14}\text{C}$ ]DXR) was studied in male Sprague-Dawley rats given 1 mg/kg body weight IV. Most of the radioactivity administered was recovered in the faeces (two-thirds of the dose within 6 days after administration), urine accounting for 15% of the  $^{14}\text{C}$  given during the same period. A significant amount of radioactivity was also found in expired air. Significantly higher levels of radioactivity were recorded in the plasma (40 min and 4 h) and liver (40 min) in [ $^{14}\text{C}$ ]DXR-treated animals, whereas in animals treated with 4'-epi-[ $^{14}\text{C}$ ]DXR a higher specific radioactivity was found in the kidneys (40 min and 4 h) and bone marrow (40 min). The total tissue residual radioactivity was greater ( $P < 0.05$ ) at 24 h for [ $^{14}\text{C}$ ]DXR (45.8%) than for 4'-epi-[ $^{14}\text{C}$ ]DXR (38.6%).

The main radioactive species in urines were the unchanged drugs. Minor metabolites were represented by a polar fraction, 13-dihydroderivatives, and aglycones. Whereas aglycones represent an important fraction of extractable tissue radioactivity in liver samples of many of the treated animals, the unchanged drug was invariably the major radioactive component in spleen, lung, and kidney. Liver extraction studies showed the presence of significant amounts of bound radioactivity that could be recovered in soluble form only after incubation with deoxyribonuclease.

The main radioactive species present in the bile were the unchanged drug and a polar fraction. The amount of the former was higher in [ $^{14}\text{C}$ ]DXR-treated than in 4'-epi-[ $^{14}\text{C}$ ]DXR-treated animals. On the other hand, partial glucuronidation of 4'-epi-[ $^{14}\text{C}$ ]DXR was deduced on the basis of results of enzymic hydrolysis of bile samples.

### Introduction

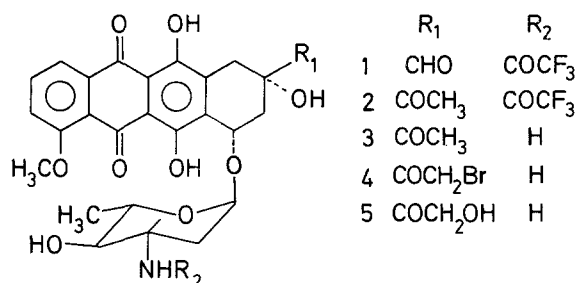
Owing to the interest 4'-epidoxorubicin (epirubicin) has aroused as a new antitumour anthracycline analogue with a lower incidence of toxic side-effects than doxorubicin, a synthesis of the  $^{14}\text{C}$ -labelled compound has been developed and its fate in laboratory animals compared with that of the parent drug. The synthesis of 4'-epi-[14- $^{14}\text{C}$ ]doxorubicinHCl (4'-epi-[ $^{14}\text{C}$ ]DXR) was achieved by a procedure similar to that successfully applied for the synthesis of [14- $^{14}\text{C}$ ]doxorubi-

cinHCl ([ $^{14}\text{C}$ ]DXR) [18]. The rat was used in this study because this rodent species has been used extensively for the evaluation of pharmacokinetic behaviour of doxorubicin [9, 13, 14, 17, 19, 22, 23–27, 31], also in comparison with man [28], and is the best known species as far as the metabolic reactions of antitumour anthracyclines are concerned [3, 11, 15, 21, 29]. Also, the rat has been used as a model for cardiotoxicity testing of doxorubicin analogues by Zbinden and Brändle [32], Philips et al. [20], Buymiski and Hirth [6], Olson [16], and others. Single doses near to the therapeutic ranges and time intervals of 40 min and 4, 24, 72, and 144 h after administration were studied. The longer time intervals were chosen to obtain an overall picture of the behaviour of the drugs and their metabolites over a period allowing almost 90% elimination of the administered radioactivity. The importance of late times is also related to the known presence of late side-effects of this type of drug, as already seen in toxicological studies.

Because of the observed high recoveries in the faeces of rats treated with 4'-epi-[ $^{14}\text{C}$ ]DXR, a study was also performed on the biliary elimination of this compound in comparison with [ $^{14}\text{C}$ ]DXR. A comparative study of biliary excretion of doxorubicin and 4'-epidoxorubicin in mice and rats has been published by Broggin et al. [5]. On the basis of fluorescence quantitative determinations 19.6% and 28.6% of the total doxorubicin dose were recovered as unchanged compound at 8 and 30 h, respectively, after treatment of cannulated rats with 10 mg/kg. At 30 h the amount of doxorubicinol excreted was 13.3%. In 4'-epidoxorubicin-treated animals the amounts of unchanged drug recovered at 8 and 30 h were 13.2% and 20%, respectively, the amount of 4'-epi-13-dihydro metabolite being 19% in the 0–30-h interval.

### Materials and methods

**Labelled compounds.** [ $^{14}\text{C}$ ]DXR was prepared according to the method described by Arcamone et al. [2], starting from [14- $^{14}\text{C}$ ]daunorubicinHCl ([ $^{14}\text{C}$ ]DNR) [18]. The radiochemical purity was 98% as determined by TLC (Silicagel Merck F 254 plates; chloroform : methanol : acetic acid : water 80 : 20 : 14 : 6 by volume) and several doses of the labelled drug with specific activity between 3.78 mCi/mmol (6.54  $\mu\text{Ci}/\text{mg}$ ) and 24.8 mCi/mmol (42.7  $\mu\text{Ci}/\text{mg}$ ) were prepared. Isotopically labelled 4'-epi-DXR was prepared by the synthetic method described above for [ $^{14}\text{C}$ ]DXR [18], starting from 9-deacetyl-9-formyl-4'-epi-*N*-trifluoroacetyl-daunorubicin Fig. 1. [ $^{14}\text{C}$ ]Diazomethane (prepared from 10 mCi, 494  $\mu\text{mol}$



**Fig. 1.** Structural formulas of 4'-epidoxorubicin (5) and its synthetic intermediates (1–4)

[<sup>14</sup>C]methylamineHCl purchased from Amersham International [4], was added, with the aid of a vacuum manifold, to 260 mg (427  $\mu$ moles) of 1 in 20 ml of a mixture of CH<sub>2</sub>Cl<sub>2</sub>/ethylether (1 : 1) by volume (for experimental details see [30]). After 60 min stirring at room temperature in vacuo the crude product was purified by preparative TLC (Silicagel Merck plates 2 mm thick; chloroform : acetone, 7 : 3 by volume), giving 44.2 mg 98% radiochemically pure 4'-epi-N-trifluoroacetyldaunorubicin 2. Pure compound 2 was submitted to a mild alkaline hydrolysis at 0° C for 60 min in 0.1 N NaOH (13 ml), and after extraction with chloroform and salification with 0.1 N methanolic HCl, yielded 40.8 mg (72  $\mu$ moles) of 4'-epi-[14-<sup>14</sup>C]daunorubicinHCl 3, 98% pure, with a specific activity of 20.1 mCi/mmol (14.5% radiochemical yield based upon [<sup>14</sup>C]methylamineHCl).

Compound 3 was transformed into 14-bromo derivative 4 by adding 10.5 mg of bromine to a solution (2 ml) of 3 in a mixture of anhydrous methanol and dioxane (1 : 1 by volume) plus ethyl orthoformate (0.05 ml) at 10° C. At the end of the reaction (about 3 h) crude 4, after precipitation from a mixture of ethyl ether/petroleum ether (1 : 2 by volume), was dissolved in 6 ml 0.25 N aqueous HBr in acetone (1 : 1 by volume) and kept at room temperature overnight. The 14-bromo derivative was then hydrolysed by adding 95 mg sodium formate to give, after extraction of the base at pH 7.5 and salification with 0.1 N HCl in methanol, 34 mg crude 4'-epi-[<sup>14</sup>C]DXR 5. An aliquot of crude 5 (5.44 mg) was purified by TLC on a silica gel plate (Merck F 254; 0.25 mm thick) with the solvent system specified above for [<sup>14</sup>C]DXR, followed by extraction of the free base from silica gel and subsequent transformation into the hydrochloride. Labelled 4'-epi-DXR 5 (1.5 mg) was obtained 97% radiochemically pure, with specific activity of 19.4 mCi/mmol (33.5  $\mu$ Ci/mg). This product was diluted with unlabelled 5 and divided into several doses in the range of 2–8  $\mu$ Ci, with a specific activity in the range of 4.8–20.1 mCi/mmol (8.3–34.7  $\mu$ Ci/mg). For the biliary excretion experiments a different batch of 5, 95.5% radiochemically pure (specific activity: 10.6 mCi/mmol or 18.2  $\mu$ Ci/mg), was used. Radiolabelled compounds for injection were formulated as follows: each dose was dissolved in aqueous NaCl solution (0.9% v/v) to a final drug concentration of 0.5 mg/ml. Amounts of this solution corresponding approximately to a dosage of 1 mg/kg were injected. The exact dosage was established by subtracting the residual radioactivity recovered in the syringe from that of the original sample.

**Animals.** Male Sprague-Dawley CD outbred rats (Charles River Italia) weighing 155–380 g were used. The animals were kept in all-glass Jencons Mark III metabolic cages from 5–10 days before treatment, and maintained on a normal laboratory

diet (Charles River type 4RF21). Drug administration was performed via the tail vein in all experiments. The animals were killed by bleeding of the abdominal aorta under ether anaesthesia at different times after drug administration. In the biliary excretion experiments, after biliary duct cannulation with a polyethylene tube (PE Clay Adams USA) under halothane anaesthesia rats were housed in single cages for at least 1 h before drug administration. The animals were allowed free movement in the cage and were given drinking water ad libitum throughout the period of bile collection.

**Determination of radioactivity.** Expired air was bubbled through a 20% (by volume) ethanolamine-methyl alcohol mixture by means of a stream of decarbonated air. Samples of ethanolamine solution and urine were taken at different time intervals and radioactivity was determined by liquid scintillation counting (LSC) on Packard model 2450 and/or 300C Liquid Scintillation Spectrometers using Rialuma (Lumac System AG, Basel, Switzerland) as LS cocktail. Faeces and large organs were lyophilized, ground, and homogenized. Triplicate samples of these materials or small organs (also lyophilized) were burnt in a Packard 306 Automatic Sample Oxidizer and counted as above. Fat containing tissues were extracted with a mixture of ether-toluene (2 : 1 by volume) and defatted tissues were treated as above. The extracts were counted separately. Radioactivity of cage washes and solids was determined and added to urinary and faecal radioactivity, respectively. Bile samples for the 0–8-h and 8–24-h intervals were collected in ice-cooled preweighed vials and their radioactivity content was measured by LS counting of a weighed aliquot (50–100 mg) in 5 ml Rialuma.

**Identification of drug and metabolites in urines.** Urine samples of treated animals were collected at predetermined times and extracted by the amberlite XAD2 column procedure according to the method of Fujimoto and Haarstad [12]. This procedure made it possible to obtain a methanolic eluate containing from two-thirds up to almost 100% of urinary radioactivity. Methanol eluates were concentrated to 0.5–1 ml under reduced pressure and aliquots of 50–100  $\mu$ l were analysed by TLC using solvent system A in comparison with standard samples of DXR, 4'-epi-DXR, 13-dihydroderivatives and DXR aglycone (Table 1). Radiochromatograph patterns were obtained with a Radiochromatoscanner Packard 7201.

**Table 1.** R<sub>f</sub> values of doxorubicin (DXR) and related compounds in thin-layer chromatography<sup>a</sup>

Compounds	A <sup>b</sup>	B <sup>b</sup>	C <sup>b</sup>
DXR	0.46	0.06	0.03
4'-Epi-DXR	0.52	0.11	0.04
13-Dihydro-DXR	0.33	0.04	0.02
13-Dihydro-4'-epi-DXR	0.33	0.06	0.03
Adriamycinone	0.96	0.84	0.68
7-Deoxyadriamycinone	0.91	0.73	0.73
13-Dihydroadriamycinone	0.76	0.61	0.38
7-Deoxy-13-dihydroadriamycinone	0.88	0.66	0.57

<sup>a</sup> Silica gel F 254 Merck plates, thickness 0.25 mm

<sup>b</sup> The following solvent systems were used: A, chloroform-methanol-acetic acid-water (80:20:14:6 by volume); B, chloroform-methanol-water (80:20:3 by volume); C, ethyl-acetate-ethanol-acetic acid-water (16:2:1:1 by volume)

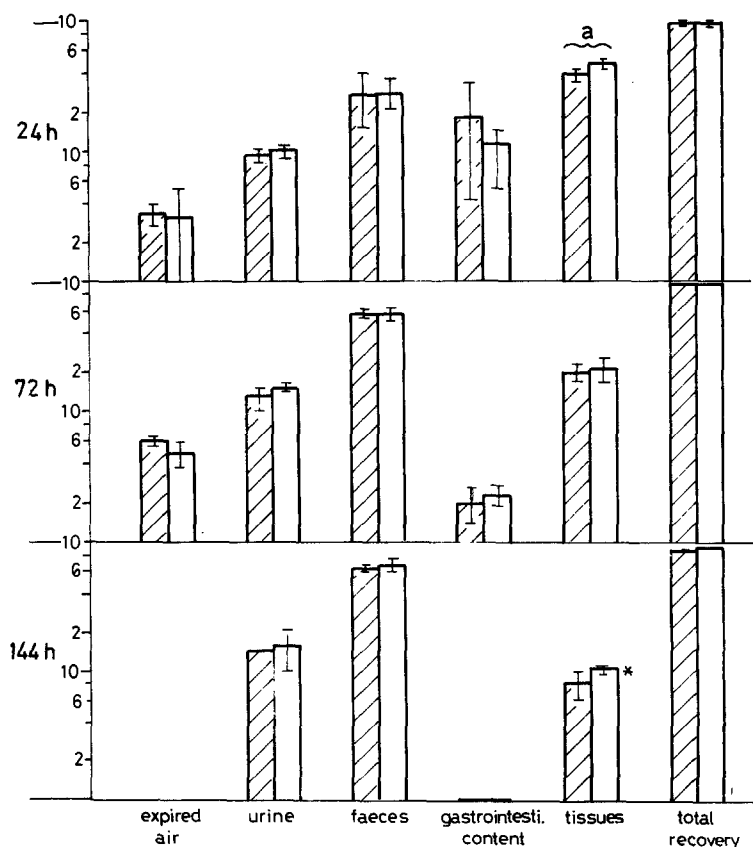
The presence of conjugates was checked in eluate samples by comparing thin-layer chromatograms before and after incubation for 16 h at 42° C with Merck  $\beta$ -glucuronidase and arylsulphatase (from *Helix pomatia*) in pH 5.4, 0.2 M acetate buffer.

**Identification of drug and metabolites in tissues.** Samples of tissue homogenates, together with 25  $\mu$ g unchanged drug and its 13-dihydroderivative in a 2:1 ratio as nonradioactive carriers, were extracted three times with a total of 20 ml aqueous 1% acetic acid. A second extraction was made with  $10 \times 7$  ml 5% acetic acid in methanol. The pooled extracts were analysed for radioactivity by LSC, while for the solids the combustion method was used. These extracts (Extract I) were evaporated under vacuum to dryness. The residue was taken up with 5% acetic acid in methanol (2–3 ml) to give a new extract (Extract II) which was analysed by TLC as described for the urine samples after volume reduction in vacuo to about 0.3 ml. The radioactivity of the remaining solid was determined by the combustion technique. Analysis of aglycone content in these extracts was performed by TLC, plates being developed in solvent systems B and C (Table 1). Adriamycinone, 7-deoxyadriamycinone, 13-dihydroadriamycinone, and 7-deoxy-13-dihydroadriamycinone were used as chromatographic standards. The radiochromatographic pattern of the aglycone fraction was determined by scraping the plates and using LSC to enumerate the areas corresponding to the above-mentioned standards.

**Exhaustive extractions.** To ascertain whether incomplete recoveries of  $^{14}\text{C}$  from tissue samples were due to DNA complexation, some extraction experiments were carried out

with a deoxyribonuclease and an acid treatment as follows. Liver homogenate samples were suspended in an equal volume of 1 M acetate buffer pH 5.4. Traces of  $\text{MgSO}_4$  and a few milligrams of deoxyribonuclease I (DNase, E.C.3.1.4.5. from bovine pancreas, Sigma Chemical Co. USA) were added and the suspension was mixed vigorously for a few minutes. Samples were incubated for 24 h at room temperature. Extraction was subsequently performed as described above to give Extract II. The solid remaining after extraction was suspended in 1 N HCl (3–4 ml) and the suspension was kept for 1 h at 90° C. After cooling the mixture was extracted with chloroform ( $3 \times 6$  ml) to give an organic extract (Extract III). The exhausted aqueous suspension was centrifuged (6,000 rpm for 15 min) twice after addition, each time, of 2–3 ml methanol to obtain a hydroalcoholic solution (Extract IV). The total radioactivity of Extracts III and IV was determined by LSC.

**Bile.** Bile samples were analysed by applying 1–10 nCi directly to semipreparative plates (Merck F 254 0.5 mm thickness with concentrating zone), and the chromatogram was developed with solvent system A (Table 1). Standard samples of 13-dihydroderivatives, unchanged drug, 13-dihydro-7-deoxyadriamycinone, 7-deoxyadriamycinone, and adriamycinone were cochromatographed with the bile samples. After drying the plates were radioscanned and areas corresponding to the different peaks were measured. Enzymic hydrolysis of biliary samples was performed according to the following procedures: (1) 100–200  $\mu$ l bile sample (corresponding to about 40–20 nCi) was treated with one tenth in volume of 6 M pH 5.4 acetate buffer. After shaking, 8–16  $\mu$ l Merck  $\beta$ -glucuronidase-arylsulphatase solution (60 U/ml and 12 U/ml, respectively)



**Fig. 2.** Balance of radioactivity after IV administration (via tail vein) of 4'-epi-[14- $^{14}\text{C}$ ]doxorubicinHCl and of [14- $^{14}\text{C}$ ]doxorubicinHCl at 1 mg/kg body weight to male Sprague-Dawley rats. Data are expressed as mean percentages of dose ( $n = 3$ ,  $\pm$  SD). Expired air not measured in 144 h animals. a,  $P \leq 0.05$ . Dashed blocks, 4'-epi-[14- $^{14}\text{C}$ ]doxorubicinHCl; clear blocks, [14- $^{14}\text{C}$ ]doxorubicinHCl. \* Mean of two animals

was added. Samples were incubated for 16 h at 42–44° C; (2) 100–200 µl bile sample was diluted with an equal volume of solution of  $\beta$ -glucuronidase (Sigma Chemical Co., 2,000 U/ml) in 0.15 M, pH 6.8 phosphate buffer and incubated for 4 h at 37–38° C. After incubation, bile samples were analysed by TLC as above.

## Results

The recovery of radioactivity in the excreta after IV administration of the labelled drugs is presented in Fig. 2. Radioactivity detectable in expired air reached a cumulative value around 5% of total dose (mean of the different experiments). The two drugs behaved almost identically, the urinary elimination being approximately 10% at day 1 and up to 15% by 6 days after treatment. Total tissue radioactivity was relatively high at 24 h and fell to approximately half the 24-h value at 72 h, a similar reduction being observed in the 72–144-h interval. Total residual tissue radioactivity found after 4'-epi-[<sup>14</sup>C]DXR treatment at 24 h was significantly lower ( $P \leq 0.05$ ) than that found after [<sup>14</sup>C]DXR treatment at the same time. Higher levels of radioactivity (Tables 2 and 3) for [<sup>14</sup>C]DXR than for 4'-epi-[<sup>14</sup>C]DXR were found in the plasma of animals sacrificed at 40 min and 4 h, the difference no longer being significant by 24 h. A similar observation can be made for the liver-specific radioactivity of animals sacrificed at 40 min. On the other hand, specific radioactivity of kidney and bone marrow tissues at 40 min was significantly higher after 4'-epi-[<sup>14</sup>C]DXR treatment, the difference being significant for the former tissue even 4 h after administration. At the later times the highest specific radioactivities were found in spleen, bone marrow, hypophysis, kidney, and liver, and the lowest in plasma, brain, and testes. Although not statistically

significant, differences between the two drugs were found in the heart tissues see also Tables 4 and 5); higher specific radioactivity was constantly found for [<sup>14</sup>C]DXR in the lung on days 1, 3, and 6 after administration. With both drugs, specific radioactivity of tissues such as liver, kidney, lung, heart, and bone marrow decreased significantly with time, starting on day 1 after administration. In contrast, thymus was found to maintain a constant high level of [<sup>14</sup>C] up to 144 h. Radioactivity of urinary extracts could be separated in four major fractions, namely unchanged drug, 13-dihydroderivative, aglycones (Rf interval 0.62–1.0) and polar unidentified metabolites (Rf interval 0.0–0.30). Cumulative excretion of the said fractions is presented in Fig. 3. Unchanged drug is the most prominent chromatographic peak in all samples. The amount of 13-dihydroderivative is lower in 4'-epi-[<sup>14</sup>C]DXR, the difference being statistically significant at 24 h. No conjugates were detectable as main constituents of the chromatographic polar fraction. The recoveries of radioactivity from the tissue samples analysed ranged from 40% to 80% in extract I, two thirds up to nine tenths of this giving rise to an homogeneous solution (Extract II) suitable for TLC analysis. Analysis of the liver extract gave the results reported in Fig. 4. In 10 of 13 animals treated with 4'-epi-[<sup>14</sup>C]DXR the major radioactive fraction was represented by aglycones. On the other hand, in the case of [<sup>14</sup>C]DXR the aglycones represent the main fraction at 40 min and 4 h, whereas the unchanged drug is the predominant species at 24 and 72 h. The composition of the aglycone fractions of liver extracts as determined in single rats is shown in Table 6. The aglycone 7-deoxy-13-dihydroadriamycinone was the main liver aglycone metabolite in all animals tested. Extracts of spleen, lung, and kidney of rats (at 4 h after treatment with 4'-epi-[<sup>14</sup>C]DXR or [<sup>14</sup>C]DXR) contained unchanged drug as the main component (from

**Table 2.** Specific tissue radioactivity<sup>a</sup> at various times after IV administration of 4'-epi-[14-<sup>14</sup>C]doxorubicin HCl to male Sprague-Dawley rats

Tissue	40 min	4 h	24 h	72 h	144 h
	Dose (mg/kg)				
	1.08 ± 0.05	0.98 ± 0.01	0.94 ± 0.22	1.01 ± 0.20	1.09 ± 0.05
Blood (in toto) <sup>b</sup>	87 ± 15 <sup>d</sup>	42 ± 6 <sup>f</sup>	32 ± 6	24 ± 3	14 ± 6
Plasma <sup>b</sup>	85 ± 19 <sup>c</sup>	42 ± 11 <sup>d, f</sup>	26 ± 10	21 ± 6	9 ± 3
Corpuscular blood <sup>c</sup>	46 ± 9	21 ± 1 <sup>f</sup>	14 ± 3	13 ± 4	8 ± 2
Liver	2,431 ± 210 <sup>e</sup>	1,259 ± 93 <sup>f</sup>	926 ± 215	668 ± 330	296 ± 150
Kidney	7,986 ± 853 <sup>e</sup>	3,254 ± 365 <sup>d, f</sup>	1,353 ± 147	741 ± 152	217 ± 110
Lung	3,404 ± 491	2,200 ± 624 <sup>f</sup>	819 ± 479	558 ± 71	225 ± 76
Spleen	5,412 ± 1,118	3,248 ± 617 <sup>f</sup>	2,695 ± 728	2,017 ± 172	974 ± 380
Heart (in toto)	2,836 ± 456	1,820 ± 160 <sup>f</sup>	725 ± 36	287 ± 52	82 ± 41
Muscle (striatum)	694 ± 23	657 ± 71 <sup>f</sup>	314 ± 44	155 ± 22	43 ± 20
Bone marrow	2,009 ± 195 <sup>d</sup>	1,721 ± 271 <sup>f</sup>	1 670 ± 319	711 ± 371	355 ± 171
Testes	71 ± 5	76 ± 12 <sup>f</sup>	72 ± 34	70 ± 11	62 ± 7
Thymus	712 ± 209	472 ± 34 <sup>f</sup>	536 ± 116	722 ± 81	580 ± 154
Hypophysis	2,835 ± 370	2,372 ± 602 <sup>f</sup>	1,647 ± 42 <sup>g</sup>	1,169 ± 146 <sup>g</sup>	617 ± 231
Skin and hair	519 ± 5	434 ± 46 <sup>f</sup>	352 ± 61	255 ± 75	175 ± 99
Intestine (tissue)	2,643 ± 699	3,331 ± 885 <sup>f</sup>	769 ± 34	338 ± 24	104 ± 35
Intestine (contents)			2,895 ± 2,016	304 ± 126	67 ± 32

<sup>a</sup> Data are expressed as mean (± SD) nanogram equivalents of antibiotic per gram of fresh tissue ( $n = 3$ )

<sup>b</sup> Read ng/ml

<sup>c</sup> Refer to ml of total blood

<sup>d</sup> Significantly different from the corresponding values for [14-<sup>14</sup>C]doxorubicin HCl ( $P \leq 0.05$ )

<sup>e</sup> As in <sup>d</sup> but  $P \leq 0.01$

<sup>f</sup>  $n = 4$

<sup>g</sup>  $n = 2$

**Table 3.** Specific tissue radioactivity<sup>a</sup> at various times after IV administration of [14-<sup>14</sup>C]doxorubicin HCl to male Sprague-Dawley rats

Tissue	40 min	4 h	24 h	72 h	144 h
	Dose (mg/kg)				
	1.06 ± 0.03	1.02 ± 0.04	1.03 ± 0.02	0.96 ± 0.17	1.02 ± 0.10
Blood (in toto) <sup>b</sup>	178 ± 11 <sup>d</sup>	67 ± 15 <sup>f</sup>	40 ± 12	21 ± 8	17 ± 2
Plasma <sup>b</sup>	185 ± 28 <sup>e</sup>	76 ± 13 <sup>d, f</sup>	40 ± 17	19 ± 8	10 ± 3
Corpuscular blood <sup>c</sup>	70 ± 9	25 ± 3 <sup>f</sup>	16 ± 3	12 ± 2	9 ± 4
Liver	4,058 ± 598 <sup>e</sup>	1,360 ± 437 <sup>f</sup>	758 ± 233	385 ± 114	263 ± 75
Kidney	4,001 ± 908 <sup>e</sup>	2,399 ± 383 <sup>d, f</sup>	1,890 ± 888	689 ± 116	249 ± 9
Lung	2,944 ± 163	2,079 ± 714 <sup>f</sup>	1,417 ± 138	939 ± 317	455 ± 241
Spleen	3,281 ± 1,002	4,055 ± 597 <sup>f</sup>	3,491 ± 202	2,158 ± 353	1,486 ± 847
Heart (in toto)	2,332 ± 190	1,568 ± 188 <sup>f</sup>	881 ± 327	318 ± 94	119 ± 41
Muscle (striatum)	697 ± 119	635 ± 158 <sup>f</sup>	474 ± 71	219 ± 76	73 ± 22
Bone marrow	1,573 ± 97 <sup>d</sup>	1,578 ± 326 <sup>f</sup>	1,756 ± 379	819 ± 138	565 ± 157
Testes	77 ± 12	87 ± 8 <sup>f</sup>	87 ± 27	92 ± 10	68 ± 6
Thymus	479 ± 114	499 ± 193 <sup>f</sup>	734 ± 190	931 ± 260	858 ± 443
Hypophysis	2,053 ± 427	1,759 ± 240 <sup>f</sup>	2,819 ± 585 <sup>g</sup>	1,260 <sup>h</sup>	817 ± 219
Skin and hair	557 ± 17	504 ± 57 <sup>f</sup>	504 ± 58	296 ± 36	236 ± 82
Intestine (tissue)	2,518 ± 840	3,402 ± 350 <sup>f</sup>	995 ± 290	355 ± 106	131 ± 41
Intestine (contents)			2,978 ± 2,892	371 ± 298	102 ± 94

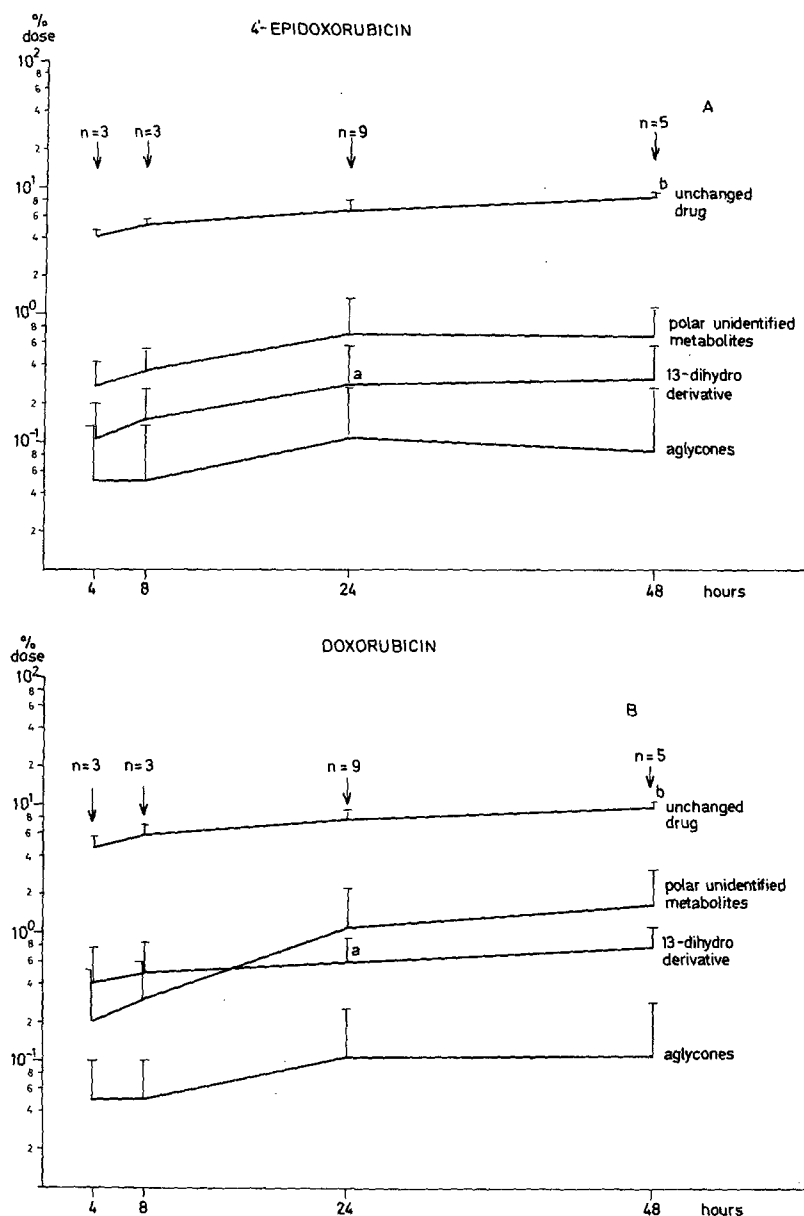
<sup>a</sup> Data are expressed as mean (± SD) nanogram equivalents of antibiotic per gram of fresh tissue (*n* = 3)<sup>b</sup> Read ng/ml<sup>c</sup> Refer to ml of total blood<sup>d</sup> Significantly different from the corresponding value for 4'-epi-[14-<sup>14</sup>C]doxorubicin HCl (*P* ≤ 0.05)<sup>e</sup> As in <sup>d</sup> but *P* ≤ 0.01<sup>f</sup> *n* = 4<sup>g</sup> *n* = 2<sup>h</sup> Single datum**Table 4.** Specific heart tissue radioactivity<sup>a</sup> at various times after IV administration of 4'-epi-[14-<sup>14</sup>C]doxorubicin HCl to male Sprague-Dawley rats

Tissue	40 min	4 h	24 h	72 h	144 h
	Dose (mg/kg)				
	1.08 ± 0.05	0.98 ± 0.01	0.94 ± 0.22	1.01 ± 0.20	1.09 ± 0.05
Left auricle	2,807 ± 643	1,920 ± 485 <sup>b</sup>	1,078 ± 219	429 ± 60	127 ± 43
Right auricle	2,513 ± 261	1,862 ± 593 <sup>b</sup>	1,171 ± 241	467 ± 107	143 ± 63
Septum	3,121 ± 225	1,859 ± 52 <sup>b</sup>	599 ± 59	242 ± 108	80 ± 40
Left ventricle	3,196 ± 469	1,862 ± 57 <sup>b</sup>	657 ± 51	289 ± 46	75 ± 36
Right ventricle	2,238 ± 445	1,667 ± 633 <sup>b</sup>	783 ± 117 <sup>c</sup>	304 ± 56	85 ± 61

<sup>a</sup> Data are expressed as mean (± SD) nanogram equivalents of antibiotic per gram of fresh tissue (*n* = 3)<sup>b</sup> *n* = 4<sup>c</sup> *n* = 2**Table 5.** Specific heart tissue radioactivity<sup>a</sup> at various times after IV administration of [14-<sup>14</sup>C]doxorubicin HCl to male Sprague-Dawley rats

Tissue	40 min	4 h	24 h	72 h	144 h
	Dose (mg/kg)				
	1.06 ± 0.03	1.02 ± 0.04	1.03 ± 0.02	0.96 ± 0.17	1.02 ± 0.10
Left auricle	2,455 ± 474	1,550 ± 438 <sup>b</sup>	1,434 ± 520	564 ± 140	224 ± 81
Right auricle	2,330 ± 201	1,351 ± 697 <sup>b</sup>	1,281 ± 313	611 ± 164	215 ± 81
Septum	2,185 ± 135	1,438 ± 497 <sup>b</sup>	816 ± 359	279 ± 80	116 ± 28
Left ventricle	2,439 ± 197	1,676 ± 110 <sup>b</sup>	832 ± 294	295 ± 85	117 ± 21
Right ventricle	2,276 ± 272	1,506 ± 318 <sup>b</sup>	914 ± 383	379 ± 106	116 ± 65

<sup>a</sup> Data are expressed as mean (± SD) nanogram equivalents of antibiotic per gram of fresh tissue (*n* = 3)<sup>b</sup> *n* = 4



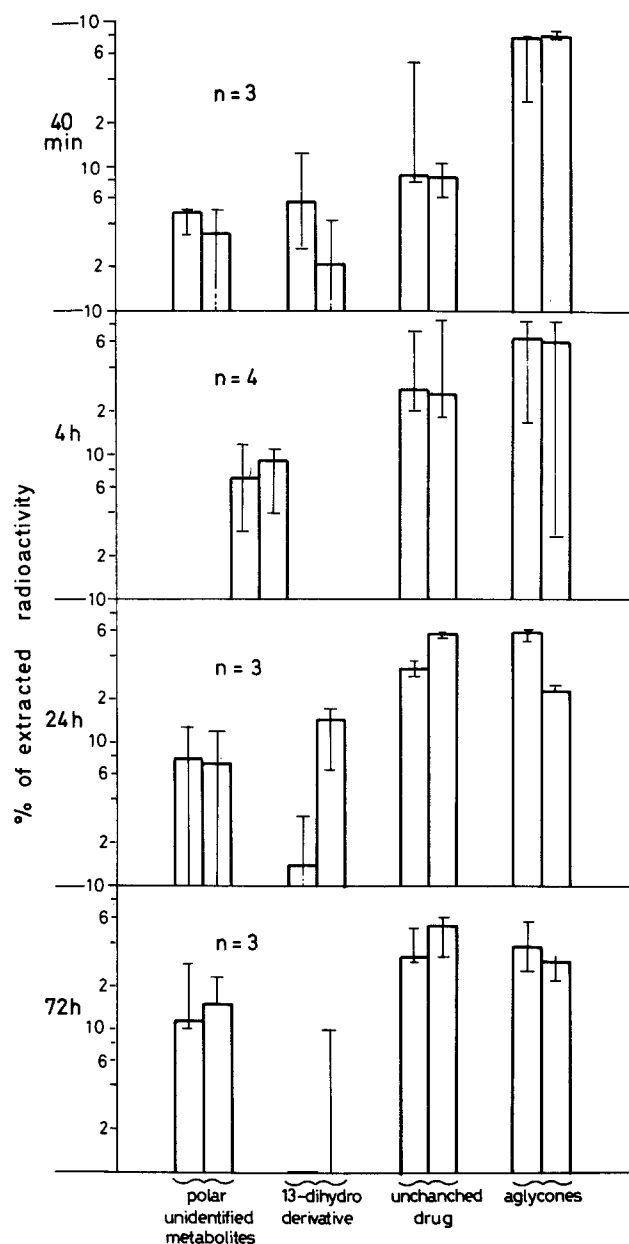
**Fig. 3A and B.** Metabolites present at different times in urine of male Sprague-Dawley rats treated IV (via tail vein) with 4'-epi-[14-<sup>14</sup>C]doxorubicinHCl (A) and with [14-<sup>14</sup>C]doxorubicinHCl (B) (1 mg/kg). Cumulative data are expressed as mean ( $\pm$  SD, for *n* see figure) percentages of dose. Statistically significant difference is indicated with a ( $P \leq 0.05$ ) or b ( $P \leq 0.10$ )

$\frac{2}{3}$  of total extracted radioactivity in kidney to  $\frac{1}{10}$  in spleen and lung) (Fig. 5). Minor components in spleen and lung were the 13-dihydroderivatives and aglycones, whereas in kidney the aglycones represent approximately one fifth of total radioactivity. Because of the incomplete recovery of radioactivity in the tissues shown above, three liver samples from doxorubicin-treated animals were submitted to an exhaustive extraction procedure, including DNase and acid hydrolytic treatments. This procedure allowed almost quantitative solubilization of sample radioactivity (Table 7). When the enzyme treatment was omitted in the exhaustive extraction procedure, lower yields were obtained in Extract II. A consistent amount of radioactive material appeared in Extract IV, that is the nonaglycone portion of the acid hydrolysate.

4'-epi-[<sup>14</sup>C]DXR and [<sup>14</sup>C]DXR behaved similarly as regards the rate of biliary elimination of total radioactivity (Table 8). For both drugs more than one third of the injected <sup>14</sup>C was recovered within 22 h after administration. Radiochromatographic analysis by TLC of biliary samples indicated the presence of a main fraction behaving as the unchanged

drug and of different metabolites at lower Rf values. Typical radiochromatograms are shown in Fig. 6. In the case of both 4'-epi-[<sup>14</sup>C]DXR and [<sup>14</sup>C]DXR the following chromatographic fractions were sufficiently resolved to allow quantitative determination: (a) loading zone residue, i.e., radioactivity present in the concentrating zone where the sample was applied; (b) polar fraction corresponding to radioactive species in the 0–0.25 Rf range; (c) radioactivity corresponding to the 13-dihydroderivative standard sample, which was accompanied in the case of 4'-epi-[<sup>14</sup>C]DXR by an adjacent unidentified peak (Rf 0.3–0.4); (d) unchanged drug. No aglycones were detected in the chromatograms of nonhydrolysed samples.

In 4'-epi-[<sup>14</sup>C]DXR-treated animals and in the 0–8 h intervals, unchanged drug represented nearly one half of the total radioactivity, a second major fraction being that containing the more polar metabolites. In the 8–22-h samples, the latter fraction, especially if considered together with the loading zone residue, accounted for the major portion of biliary radioactivity. In [<sup>14</sup>C]DXR-treated animals the amount of unchanged drug was in general clearly higher than in the



**Fig. 4.** Analysis of liver extracted radioactivity at different times after IV administration of 4'-epi-[14-<sup>14</sup>C]doxorubicinHCl (*left*) and [14-<sup>14</sup>C]doxorubicinHCl (*right*) (1 mg/kg) to male Sprague-Dawley rats. Data are expressed as percent median values (and range) for extracted radioactivity. No resolution of the more polar peaks occurred in the 4-h samples

corresponding 4'-epi-[<sup>14</sup>C]DXR samples. Also in this case the relative amount of polar metabolites increased in the bile collected after 8 h from the time of treatment. Similarly to what was observed in the rat urinary excretion studies, the relative amount of 13-dihydroderivative was definitely higher in [<sup>14</sup>C]DXR-treated than in 4'-epi-[<sup>14</sup>C]DXR-treated animals.

For evaluation of the presence of conjugates, bile samples were submitted to enzymatic hydrolysis using procedures 1 and 2. Procedure 1 was discarded because of the evident degradation when the drug itself was incubated in the same conditions and analysed by radio TLC. Degradation was in the order of 20% and more polar products were detected in a broad zone of the chromatogram. In the case of 4'-epi-[<sup>14</sup>C]DXR one degradation product showed the same R<sub>f</sub> value as the unidentified peak of the bile sample mentioned above. When procedure 2 was used, the chromatograms shown in Fig. 6 were obtained. In the bile of the 4'-epi-[<sup>14</sup>C]DXR-treated animals a decrease of the polar fractions was accompanied by an increase (from 46% to 60% of sample radioactivity) of unchanged drug. This suggests the presence of a glucuronide of 4'-epi-[<sup>14</sup>C]DXR. In contrast, in the bile of the [<sup>14</sup>C]DXR-treated animal the amount of unchanged drug after hydrolysis (56% of sample radioactivity) was practically the same as that found before the enzymatic treatment (58% of sample radioactivity). However, small amounts of aglycone-type metabolites were revealed by radiochromatography after enzymatic hydrolysis.

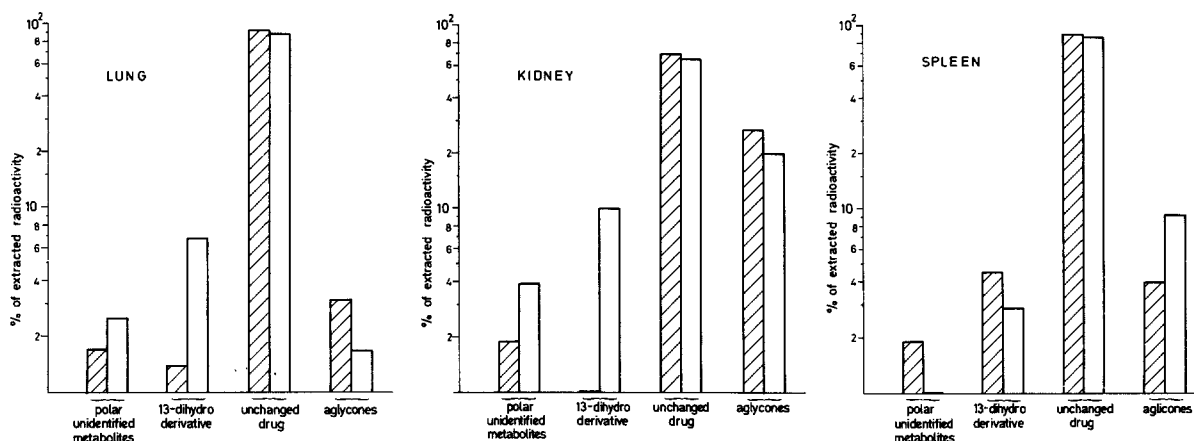
## Discussion

The similar behaviour observed in the elimination of the two drugs is in agreement with the slight difference in the chemical structure of the two compounds. The same is also true for the total radioactivity retained in the tissue, although this value is somewhat lower in the case of 4'-epi-[<sup>14</sup>C]DXR than with [<sup>14</sup>C]DXR the difference being significant only at 24 h, however. A more rapid elimination of 4'-epi-[<sup>14</sup>C]DXR than of [<sup>14</sup>C]DXR has been recorded in tumour-bearing mice and in normal animals according to a total fluorescence method of analysis [7, 8]. Our results concerning urinary excretion of doxorubicin are only partially comparable with literature data, because therapeutic doses were used in the present study whereas higher doses (often > DL<sub>10</sub>) were employed in the other published investigations [14, 22–24, 31]. However, good agreement is found with the other studies indicating urinary excretion as a minor elimination route in normal rats [14, 23, 24, 31], whereas a much higher value was recorded by Sonneveld and Van Bekkum [22]. The last-named authors

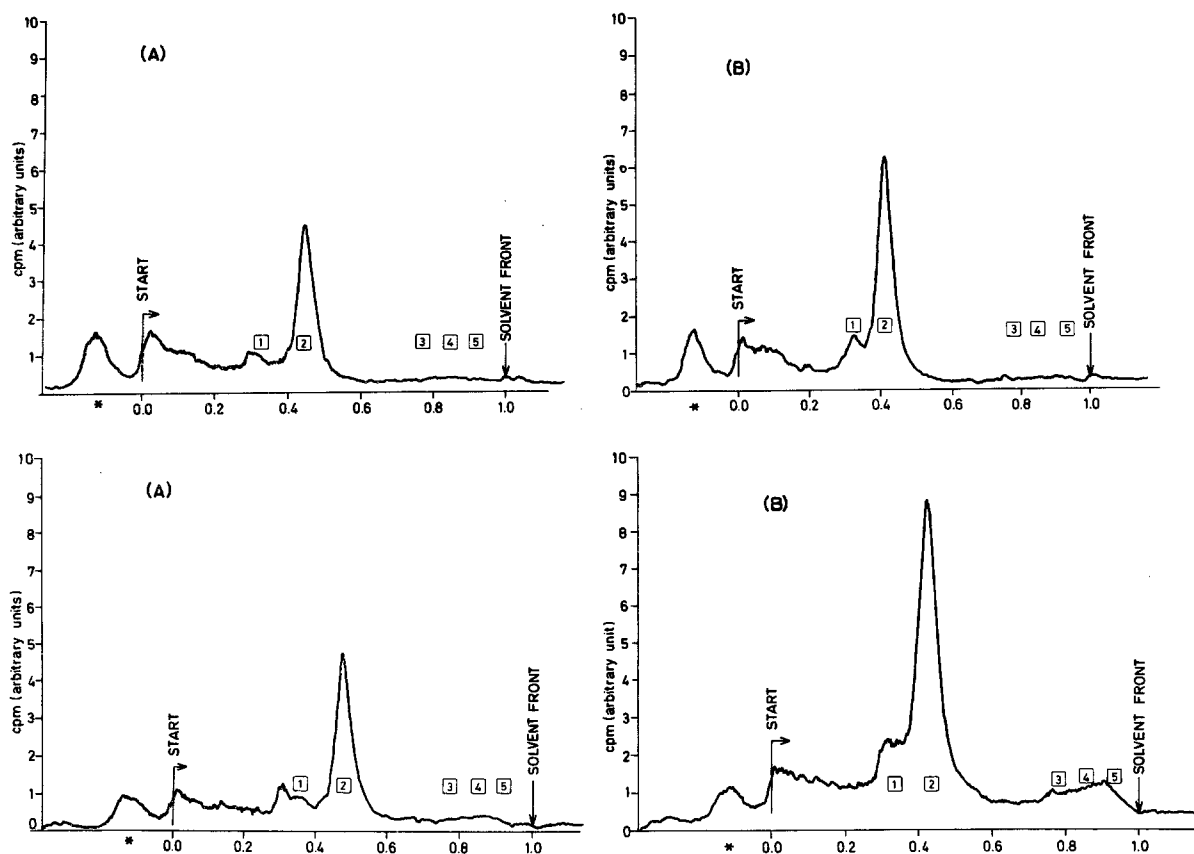
**Table 6.** Aglycones<sup>a</sup> in liver extracts of single rats at different times after treatment

	Compound							
	4'-Epidoxorubicin				Doxorubicin			
	40 min	4 h	24 h	72 h	40 min	4 h	24 h	72 h
13-Dihydroadriamycinone	3.0	5.8	13.4	6.3	5.3	6.6	10.3	7.3
7-Deoxy-13-dihydroadriamycinone	87.9	82.6	60.4	71.6	82.9	74.1	72.7	81.3
Adriamycinone	7.2	6.7	3.3	13.8	7.6	12.8	0.0	3.9
7-Deoxyadriamycinone	1.9	4.9	22.9	8.3	4.2	6.5	17.0	7.5

<sup>a</sup> Data are expressed as percentages of the total radioactivity that are present in the aglycone fraction



**Fig. 5.** Analysis of spleen, lung, and kidney radioactivity extracted at 4 h after IV administration of 4'-epi-[14-14C]doxorubicin HCl (*dashed blocks*) and [14-14C]doxorubicin HCl (*clear blocks*) (1 mg/kg) to male Sprague-Dawley rats. Data are from single animals and are expressed as percentages of extracted radioactivity



**Fig. 6A and B.** Typical radiochromatograms of 0–8-h bile samples from rats treated with 4'-epi-[14-14C]doxorubicin HCl (A) or [14-14C]doxorubicin HCl (B) before (*above*) and after (*below*) enzymic hydrolysis. Chromatographed compounds were: (1) 13-dihydroderivative, (2) unchanged drug, (3) 13-dihydro-7-deoxyadriamycinone, (4) 7-deoxyadriamycinone, (5) adriamycinone. \* Loading zone

used a different strain of rats from the one used in the present study, and the 'total fluorescence' method of analysis. As regards the expired radioactive CO<sub>2</sub>, our data are in close agreement with those recorded by Liss et al. [14] with [14-14C]DXR. The same can be said for radioactivity found in the faeces, which was 65% of the dose at 96 h according to the last-mentioned authors. Total tissue residual radioactivity was not measured in the cited studies and therefore no comparison

can be made of our data with those reported in literature for DXR. The lower plasma level of radioactivity following 4'-epi-[14-14C]DXR administration than after [14-14C]DXR is in agreement with similar observations recorded by Broggin et al. [5] in tumour-bearing mice treated with a much higher dose. Levels of total radioactivity in individual rat tissues after doxorubicin administration differ considerably from the value found by Wilkinson and Mawer [28], but are in substantial



**Table 7.** Exhaustive extraction study to examine the effect of DNAase incubation on the recovery of radioactivity<sup>a</sup> in extracts II, III, and IV of liver samples from rats treated with [<sup>14</sup>C]doxorubicin HCl

Sample (animal)	DNA-ase	Extract			
		II	III	IV	Total
RA16 <sup>b</sup>	Yes	67.9	4.2	27.7	99.8
	No	49.7	10.8	34.6	95.1
RA21 <sup>b</sup>	Yes	97.3	2.0	0	99.3
	No	49.7	ND	ND	ND
RA22 <sup>c</sup>	Yes	82.6	2.3	16.2	101.1
	No	66.6	6.9	24.3	97.8
RA22 <sup>c</sup>	Yes <sup>d</sup>	81.7	2.2	23.1	107.0
	No <sup>d</sup>	67.0	5.7	14.0	86.7

<sup>a</sup> Results are expressed as the percentages of the total radioactivity present in the sample

<sup>b</sup> Animals sacrificed at 4 h

<sup>c</sup> Animals sacrificed at 24 h

<sup>d</sup> Incubation for 96 h

**Table 8.** Biliary excretion of radioactivity<sup>a</sup> following a single IV administration of 4'-epi-[<sup>14</sup>C]doxorubicin HCl and [<sup>14</sup>C]doxorubicin HCl to male Sprague-Dawley rats at 1 mg/kg b.w.

Time intervals (h)	4'-Epidoxorubicin	Doxorubicin
0–8	23.91 ± 3.61	30.06 ± 1.13
8–22	11.35 ± 0.71	8.53 ± 1.10
0–22	35.26 ± 3.76	38.59 ± 1.22

<sup>a</sup> Results are expressed as mean (± SD) percentages of injected radioactivity (*n* = 3)

agreement with those reported by Yesair et al. [31] and by Sonneveld and Van Bekkum [22]. Comparison with other studies concerning 4'-epi-DXR [5, 7] is difficult because of the different animal species and methodology used (fluorescence versus total radioactivity). The significant difference found at the earlier times between the two drugs as regards the specific radioactivity of kidney and bone marrow is accompanied by an overall trend of the epi derivative to give higher concentrations in nearly all tissues, an important exception being the liver. This difference between the two drugs, which is evident at 40 min tends to disappear at 4 h, whereas an opposite relationship appears to be established at 24 h and at later times. The higher concentrations of [<sup>14</sup>C]DXR with respect to 4'-epi-[<sup>14</sup>C]DXR in heart auricles and in the lungs is noteworthy (albeit not statistically significant). These data agree with similar differences reported by other authors [7, 8]. The presence of the 13-dihydroderivative as a metabolite of 4'-epi-[<sup>14</sup>C]DXR, also observed in previous animal studies [5], suggests a metabolic pattern qualitatively similar to that of the other clinically used anthracyclines, namely daunorubicin and doxorubicin [1]. The low amount of the reduced metabolite found in urine indicates that 4'-epi-[<sup>14</sup>C]DXR, like [<sup>14</sup>C]DXR itself, differs from daunorubicin, which is more extensively metabolized by the enzyme aldo-ketoreductase [10]. Partial extraction of radioactivity from tissues yields somewhat

questionable tissue levels measured by fluorescence methodology. Data provided by such 'cold procedures' should certainly be considered as of lower value than actual values of total drug equivalents present in the tissues.

The strong binding of unchanged drug (or its metabolites) to cell DNA could account for the observed phenomenon. In fact the exhaustive extraction experiments clearly show that two forms of [<sup>14</sup>C]DXR-derived radioactivity that are not readily extractable are present in liver tissues. The first, ranging from 16% to 50% of the total sample radioactivity, is attributed to tightly DNA-bound drug and/or metabolites. This observation is in agreement with the known high affinity of DXR and related compounds with double-stranded native DNA [1]. The second, namely the non-chloroform-extractable portion of acid hydrolysate, might be related to acid degradation products of cell constituents covalently bound to radioactive drug or metabolites. Alternatively, this fraction might represent small-molecular-weight radioactive compounds arising from side chain degradation of administered [<sup>14</sup>C]DXR. This second hypothesis would be supported by the recovery of <sup>14</sup>CO<sub>2</sub> in expired air from all animals tested. The greater importance of the aglycone fraction in liver extracts following 4'-epi-[<sup>14</sup>C]DXR than after [<sup>14</sup>C]DXR is in agreement with the observation of Broggin et al. [5], who found lower levels of native drug, in terms of total-liver extractable fluorescence, for 4'-epiDXR than for DXR. The lower persistence of 4'-epiDXR in unchanged form may in part explain the lower toxic side-effects of the analogue. Similar conclusions can be drawn from the analysis of biliary samples. Although 4'-epi-[<sup>14</sup>C]DXR appears to be the major nonconjugated compound extracted in the bile it appears to be metabolized to a larger extent than [<sup>14</sup>C]DXR. The presence of glucuronidated forms of the unchanged drug in the case of 4'-epi-[<sup>14</sup>C]DXR represents, at least qualitatively, an important difference from its parent compound [<sup>14</sup>C]DXR.

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