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Radioactive species in rat urines and tissues after [^{14}C] AD 32 administration

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Summary. *[14-^{14}C]N-Trifluoroacetyldoxorubicin-14-valerate ([^{14}C]AD 32) was synthesized and administered IV to male rats at 9.09 mg/kg. Urinary radioactivity excreted in the 0–24 h interval was only 2.3% of the dose, N-trifluoroacetyldoxorubicin (AD 41), 13-dihydro-N-trifluoroacetyldoxorubicin (AD 92), and doxorubicin being the major urinary metabolites identified. Doxorubicin and AD 32 were the main radioactive species extracted from tissues at 24 h after treatment. The amount of doxorubicin present in the analysed tissue samples is in agreement with the relatively low toxicity of AD 32 compared with doxorubicin.*

Introduction

N-Trifluoroacetyldoxorubicin 14-valerate (AD 32) is a doxorubicin derivative showing outstanding antitumour activity in experimental tumours in mice [4], optimal doses, however, being 10–30 times those of the parent drug [1, 4, 11]. Although the new derivative was found to have no appreciable affinity for DNA [13] and to show a different intracellular distribution [9], it caused inhibition of incorporation of [^{3}H]thymidine in different tissues of L1210-bearing mice similar to that shown by a 10 fold lower dose of doxorubicin. Also, the screening data in different murine experimental models do not allow differentiation between the antitumour properties of AD 32 and doxorubicin [1]. Because of the availability of a convenient method for the synthesis of [14-^{14}C]doxorubicin derivatives [10], we prepared AD 32 labelled in the side chain; other authors have used radioactive drug in which the label is located in the trifluoroacetyl group [7, 8]. The radioactive compound was administered to rats and the results of analytical determinations concerned with the metabolic fate of AD 32 are reported here.

Materials and methods

[14-^{14}C]*N*-Trifluoroacetyldoxorubicin-14-valerate ([^{14}C]AD32) was prepared according to the following sequence. [14-^{14}C]daunorubicin hydrochloride (90 μmoles, 5.3 mCi/mmole) prepared as already described [10] was converted to the 14-bromoderivative (69% yield), which was *N*-trifluoroacetylated and subsequently submitted to nucleophilic substitution to give crude [^{14}C]AD32. Analytically pure substance was obtained by TLC preparative chromatography (2 mm silica gel, Merck), with system $CHCl_3$: MeOH (92 : 8 v/v) as the eluting solvent. The product yield amounted to 21.5 μmoles and its radiochemical purity was ≥ 97% as checked by TLC analysis using silica gel F 254 0.25-mm Merck plates in solvent system CHC_3 : MeOH (8 : 2 v/v).

Three male CD-COBS rats (Charles River – Italy) weighing 200 ± 5 g (mean ± SD) received injections IV into the tail vein with [^{14}C] AD 32 formulated in 2.8% cremophor EL (BASF FRG), 8.3% ethyl alcohol, and 88.9% isotonic saline solution at a dose of 9.09 ± 0.13 (n = 3 ± SD) mg/kg/body weight. Single urine samples were collected at 5 and 24 h after drug administration. Animals were sacrificed at 24 h by bleeding of the abdominal aorta under ethyl ether anaesthesia. Radioactivity levels in faeces, organs, and tissues were determined by the combustion technique (Packard oxidizer model 306). Radioactivity in urine samples was determined by liquid scintillation counting.

Urine metabolites were extracted according to the method of Fujimoto and Haarstad [3]. Enzymatic hydrolysis of methanol urinary extracts was performed with beta-glucuronidase and arylsulphatase (12 and 60 U/ml, respectively, Merck) at pH 5.4 in acetate buffer 0.2 *M* (50 μl for each sample of about 20 ml). The hydrolysate was reextracted by the XAD-2 absorption procedure [3]. The metabolic pattern was determined on methanolic resin eluates before and after enzymatic hydrolysis by radio TLC (silica gel plate 5 × 20 0.25 mm F 254 Merck) in solvent system A ($CHCl_3$: MeOH : H_2O, 80 : 20 : 3 v/v) and B ($CHCl_3$: MeOH : AcOH : H_2O, 80 : 30 : 3 : 3 v/v) in comparison with standard samples of AD 32, *N*-trifluoroacetyldoxorubicin (AD 41), 13-dihydro *N*-trifluoroacetyldoxorubicin (AD 92), 7-deoxy doxorubicin aglycone and doxorubicin (DXR). (Unlabelled reference compounds were kindly provided by S. Penco and G. Franchi.) Metabolites in liver, kidney, spleen, and lung were extracted as described by Schwartz [12] and analysed by TLC in system A.

Results

Urinary excretion accounted only for a small percentage of the administered dose (Table 1), as already found in the mouse by fluorimetric determinations [5]. The faecal route of elimination was the major one (78.4 ± 7.3% of dose, n = 3, ± SD) for the 0–24 h time interval. This figure is in agreement with the biliary excretion found in rats in the same time interval by Israel et al. [6]. Total recovery of radioactivity at 24 h accounted for about 90% of the administered dose, including 2.3% of the dose recovered in total body tissues.

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Table 1. Radioactive fractions (as percentage of urine sample)[a] recovered in 0–5- and 5–24-h urine samples before and after enzymic hydrolysis

Urine sample		Enzymic hydrolysis	Radioactive fractions[c]					
Time interval (h)	% of dose		AD 32	AD 41	AD 92	DXR	UM[d]	Polar metabolites
0–5	1.15 ± 0.24	No	0.0	22.4 ± 4.0	17.9 ± 0.6	6.7 ± 1.0	0.0	33.2 ± 5.4
		Yes	0.0	28.6 ± 2.8	18.2 ± 5.0	7.2 ± 3.3	4.1 ± 3.6	13.0 ± 5.5
5–24	1.19 ± 0.35	No	0.0	5.5 ± 0.5[b]	5.4 ± 1.7	4.6 ± 1.4	0.0	45.9 ± 2.8
		Yes	0.0	8.6 ± 2.8[b]	16.7 ± 3.3	2.5 ± 2.1	14.4 ± 4.6	14.1 ± 4.2

[a] Data are given as means ± SD (n = 3)
[b] Aglycone derivatives may be present
[c] Radioactivity not analysed represents the material not adsorbed on XAD2
[d] Unknown metabolite(s) in a broadened band between doxorubicin and AD 92

Table 2. Radioactivity levels (ng eq) of *N*-trifluoroacetyldoxorubicin-14-valerate per gram of fresh tissue at 24 h after IV administration of [14-^{14}C] *N*-trifluoroacetyldoxorubicin-14-valerate to male Sprague-Dawley rats (dose 9.09 ± 0.13 mg/kg)

	ng/g ± SD		n
Blood	469	101	2
Plasma	247	88	3
Corpuscular blood	362	120	2
Liver	1,226	317	3
Kidney	701	196	3
Lung	528	352	3
Spleen	1,631	104	3
Heart	249	99	3
Left auricle	388	267	3
Right auricle	390	211	3
Septum	236	86	3
Left ventricle	234	84	3
Right ventricle	246	88	3
Muscle	101	28	2
Bone marrow	687	395	3
Testes	85	25	2
Thymus	918	412	3
Hypophysis	541	180	3
Brain	46	15	2
Submaxillary glands	372	153	3

AD 41, AD 92, and DXR were present in urines collected in the 0–5 and 5–24-h time intervals, in the ratios of 3 : 2 : 1 and 1 : 1 : 1, respectively (Table 1). They accounted for about 47% and 15% of total urinary radioactivity at the time intervals examined, while the remaining portion of analysed radioactivity consisted of polar (unidentified) metabolites. No AD 32 has been identified in the urinary samples. After enzymic hydrolysis polar unidentified metabolites appear to account for about one-seventh of the total urinary radioactivity. AD 41, AD 92, and DXR changed their ratios to 4 : 2 : 1 and 4 : 7 : 1 for the 0–5- and 5–24-h time intervals, respectively. No AD 32 was identified after hydrolytic treatment at either time points. An unknown metabolite (UM) accounting for 4.1% and 14.4% of total urinary radioactivity at 0–5 and 5–24 h, respectively, appeared after enzymic hydrolysis as a broad peak of intermediate polarity between DXR and AD 92. After enzymatic hydrolysis, AD 41 increased in the urinary extract from the 0–5-h interval, thus becoming the main radioactive species (about 40% of analysed radioactivity). Conversely, in the 5–24-h time interval AD 92 was the main metabolite (it accounted for about 30% of analysed radioactivity), while small quantities of aglycones might be present both before and after enzymatic hydrolysis.

At 24 h after administration highest radioactivity levels were found, in declining order, in spleen, liver, thymus, kidney, bone marrow, hypophysis, and lung, the specific radioactivity in the lung being about one-third that in the spleen (Table 2). The lowest levels were found in brain, testes, and muscle, and heart auricles retained higher radioactivity levels than ventricles. Because of the high levels of radioactivity, samples of liver, kidney, spleen, and lung were extracted and analysed. DXR and AD 32 were found to be the major radioactive species present in the extracts (Table 3). Their ratio ranged from 2 : 1 in kidney to 1 : 1 in liver, other metabolites (about 20% of extract) also being present. On the other hand, spleen and lung contained DXR and AD 32 only in approximately equal amounts.

Discussion

The availability of radioactive AD 32 labelled at position 14 has allowed the radiochemical quantitation of all anthracycline derivatives originating from the in vivo transformation of the parent drug. This was not the case with the compound labelled in the *N*-trifluoroacetyl group used by other authors [7, 8], as our results indicate that *N*-deacylation is one of the reactions involved in the metabolism of AD 32 in vivo.

Urinary excreted radioactivity expressed as a percentage of a dose is almost five times lower than that observed after administration of [^{14}C]doxorubicin, since for the latter 10% of the dose was recovered in the 0–24-h urinary sample [2]. The lower urinary excretion can be tentatively related to the higher lipophilicity and different molecular weight of AD 32 compared with doxorubicin. On the other hand, the particularly high faecal excretion in the same time interval ([^{14}C]doxorubicin 27.5% [2]) is a typical property of AD 32 that appears to be a rapidly eliminated anthracycline derivative. In fact the amount present in body tissues after 24 h is only about 50% of the total radioactivity present 24 h after the administration of [^{14}C]doxorubicin, 1 mg/kg [2]. This observation is in agreement with the median ratio (range 1 : 6–1 : 35) of the optimal doses of the two drugs in mice bearing different tumours [1]. The presence of AD 41 and AD 92 and also of polar metabolites in

Table 3. Chromatographic distribution of radioactivity in extracts of liver, kidney, spleen, and lung of male Sprague-Dawley rats 24 h after IV injection of [14-^{14}C] *N*-trifluoroacetyldoxorubicin-14-valerate (dose 9 mg/kg)

Organs	Radioactivity levels[a] (ng/g)	% of tissue radioactivity extracted	Mean percentage of extracted radioactivity (± SD, $n = 3$)		
			AD 32	DXR	Other metabolites[b]
Liver	1,226 ± 317	30.4 ± 12.1	41.3 ± 3.8	38.3 ± 5.5	20.3 ± 4.7
Kidney	701 ± 196	24.3 ± 6.6	29.0 ± 1.0	57.0 ± 1.7	13.7 ± 0.6
Spleen	1,631 ± 104	35.0 ± 5.1	54.3 ± 3.2	45.7 ± 3.2	0.0
Lung[c]	678 ± 58	40.0 ± 1.9	38.5 ± 4.0	61.5 ± 4.0	0.0

[a] Expressed as nanogram equivalents of *N*-trifluoroacetyldoxorubicin 14-valerate per gram of fresh tissue
[b] Broadened band including 7-deoxydoxorubicin aglycone, AD 41, and AD 92
[c] $n = 2$

the urine is in agreement with the results of Israel et al. in biliary cannulated rats [6]. A novel finding is the recovery of a noticeable amount of doxorubicin clearly derived from AD 41 upon *N*-deacylation. The amount of doxorubicin recovered in the 24-h urine, corresponding to 0.13% of administered AD 32, is the amount that would be expected in the 0–24-h interval if 0.16 mg/kg of doxorubicin were injected at time 0. Conjugation appears to be an important step in AD 32 disposal. Doxorubicin is not present in conjugated form, whereas the *N*-trifluoroacetylated metabolites do seem to be. No information is available on the structure of polar metabolites and on the so-called unknown metabolite(s) whose presence is evident in the low Rf region of TLC plates after hydrolysis.

The pattern of distribution of radioactivity in rat tissues is of interest, expecially when compared with the corresponding pattern after [14-^{14}C]doxorubicin treatment [2]. The blood radioactivity, which is about the same or even higher than the radioactivity found in the organs (with the exception of spleen, liver, thymus, and kidney), suggests a much lower fixation of AD 32 and its major metabolites than of doxorubicin to body tissues. On the other hand, the high concentration in the liver is in agreement with the importance of biliary excretion of AD 32-derived radiolabelled species. It may be noted that for most tissues the level of radioactivity is 0.2–0.5 times that found after administration of doxorubicin at a nine times lower dose. These observations offer an explanation for the lower toxicity of AD 32 than of doxorubicin.

A comment can be made on the tissue extraction studies concerning the low amount of extractable radioactivity (which is in the range of 24%–40% of total tissue radioactivity). Similar values have been recorded with the extraction of liver samples from [^{14}C]doxorubicin-treated rats [2]. The low recovery has been attributed to the high affinity of doxorubicin for cell DNA. In the case of AD 32 the low recovery data are somewhat surprising, because of the virtual absence of interaction with DNA [13] and because of the lipophilicity of the compound. The conclusion should be that the nonextractable radioactivity, about 72% of total tissue radioactivity, is due to radioactive species different from AD 32. That these species might include doxorubicin itself is suggested by the presence of doxorubicin as a major component of tissue extracts.

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