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Distribution of Radioactivity and Anthracycline-fluorescence in Tissues of Mice One Hour After [^{14}C]-Labeled AD 32 Administration

Evidence for Tissue Aglycone Formation

Mervyn Israel, Abraham M. Karkowsky, and Vinod K. Khetarpal

Division of Pharmacology, Sidney Farber Cancer Institute, 44 Binney Street, Boston, MA 02115, USA

Summary. *Levels of radioactivity and total anthracycline fluorescence in tissues of A/JAX mice were compared 1 h after IV administration of unlabeled or [^{14}C]-labeled AD 32 (50 mg/kg). Highest levels of both fluorescence and radioactivity were found in the small intestine (including contents) and liver, a result consistent with the known hepatobiliary excretion of AD 32 and metabolites. Significant accumulations of radioactivity and fluorescence were found in kidney, spleen, large intestine (including contents), lung, and heart. Lesser levels were found in muscle and fat. Little radioactivity and fluorescence were found in brain. Liquid chromatographic analysis of extracts of small intestine and liver homogenates showed N-trifluoroacetyladriamycin (AD 41) as the major fluorescent species, and also revealed N-trifluoroacetyladriamycinol (AD 92) and occasional low levels of AD 32. In addition, there was a major peak of nonfluorescent radioactive material and two fluorescent nonradioactive signals (unknowns 1 and 2), indicative of cleavage of the radiolabel from the chromophore.*

Introduction

AD 32, a disubstituted analog of adriamycin (ADR), is presently undergoing clinical trial [1] based upon its therapeutic superiority in murine tumor systems [5, 12, 13] to the parent antibiotic and its lower toxicity [3] in animal model test systems. Previous studies in mice [7], rats [8], monkey [10], and humans [2, 11] have shown that AD 32 undergoes extensive biotransformation, as measured by metabolites isolated from biological fluids. The major metabolites, N-trifluoroacetyladriamycin (AD 41) and N-trifluoroacetyladriamycinol (AD 92), still contain the intact N-trifluoroacetyl group. Little or no ADR was detectable in plasma, bile and/or urine in these earlier studies. These findings led us to prepare a sample of AD 32 bearing a [^{14}C]-label on the carbonyl carbon atom of the trifluoroacetyl moiety. Since earlier work quantitating AD 32 and metabolites was based upon fluorescence analysis, one intended use of this radiolabeled preparation was for the detection and monitoring of possible nonfluorescent radioactive metabolites of AD 32. Indeed, when levels of radioactivity and total anthracycline fluorescence in plasma and urine from mice were compared following the administration of AD 32, data suggested that AD 32 was partially converted into previously unrecognized nonfluorescent metabolite(s) [9]. The present study compares radioactivity and anthracycline fluorescence in tissues of mice 1 h after administration of a dose of AD 32. This time point was selected because by 1 h post injection drug distribution into tissue spaces is virtually completed ($T_{1/2}\alpha \sim 3$ min) but significant elimination of metabolites has not yet occurred [9]. Anthracycline species present in different tissues at this time point would, therefore, provide evidence for the metabolic processing of drug in these tissues.

Materials and Methods

Chemical Agents. Unlabeled AD 32, prepared for us by Farmitalia, Milan, Italy (lot no. 70440 E-396), was kindly provided by Adria Laboratories Inc., Columbus, Ohio, USA. Drug was formulated at a concentration of 3.5 mg/ml in 5% Emulphor-5% ethanol-90% saline (0.9% NaCl w/v), as previously described [9]. Emulphor, a polyethoxylated castor oil derivative, was supplied as a 1:1 concentrate with ethanol by J. Paul Davignon, Pharmaceutical

Reprint requests should be addressed to: M. Israel

The abbreviations used are: AD 32, N-trifluoroacetyladriamycin-14-valerate; AD 41, N-trifluoroacetyladriamycin; AD 92, N-trifluoroacetyladriamycinol; ADR, adriamycin; AD 48, adriamycin-14-valerate; AD 60, 13-dihydro-N-trifluoroacetyladriamycin-14-valerate; AMNOL, adriamycinol; HPLC, high-performance liquid chromatography; TLC, thin layer chromatography; SDS, sodium dodecylsulfate

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Resources Branch, Division of Cancer Treatment, Bethesda, MD, USA.

The preparation of radiolabeled AD 32 from AD 41 and [1-^{14}C]-trifluoroacetic anhydride, and its formulation in the Emulphor vehicle, have been reported [9].

AD 41, AD 92, AD 48, and AD 60, used as standards for HPLC retention time analysis, were prepared in this laboratory [4, 6]. Bulk ADR hydrochloride, which was used for the preparation of AMNOL, and also for the determination of extraction efficiency and as an HPLC reference standard, was kindly provided by Farmitalia, Milan, Italy. AMNOL was prepared from ADR by potassium borohydride reduction in the cold [6].

Determination of Radioactivity and Total Anthracycline Fluorescence in Mouse Tissues Following Treatment with AD 32. Male A/JAX mice (Jackson Laboratories, Bar Harbor, Maine, USA) weighing 16–29 g received injections into the tail vein of either unlabeled AD 32 (50 mg/kg) or radiolabeled AD 32 (50 mg/kg, ~ 175,000 cpm), and were sacrificed 1 h later. Tissues were immediately excised, rinsed in ice-cold isotonic saline, blotted dry, weighed, and prepared for extraction as described below. Extraction procedures for tissue samples were the same whether labeled or unlabeled drug was injected into the animal.

Tissues were homogenized with Tris-HCl buffer (0.05 *M*, pH 8.5, containing 3% w/v SDS) (9 ml/g tissue) in Duall ground-glass tissue grinders (Kontes Glass Co., Vineland, NJ, USA). The entire homogenate, or an aliquot, was extracted three times with two volumes of ethyl acetate/1-propanol (9 : 1 by volume) for each volume of homogenate. When radiolabeled AD 32 was injected into the mouse, the combined organic extracts were transferred to a scintillation vial, Aquasol scintillation fluid (New England Nuclear, Inc., Boston, MA, USA) was added, and the radioactivity was counted on a Beckman LS-335 liquid scintillation counter. When unlabeled AD 32 was injected, the combined organic extracts were evaporated to dryness at 45° C in a vortex evaporator (Buchler Instruments, Fort Lee, NJ, USA). The residue was dissolved in 3 ml methanol, and 0.2 ml Tris-HCl buffer (0.5 *M*, pH 8.4) was added. Samples were then centrifuged to remove turbidity and were read on a Perkin-Elmer Model MPF-4 corrected spectrum spectrofluorometer. Tissues from animals treated with solvent only served as blanks for fluorescence assays.

The values observed for tissue levels of anthracyclines with either radioactivity or fluorescence were compensated for by the effect of tissue extract quenching. In one set of radioactivity experiments [^{14}C]-toluene was used as an internal standard. Quenching in the radioactivity experiments was found to be uniformly small for all tissues studies (approximately 4%), and a factor of 1.04 was therefore applied to correct observed radioactivity to actual radioactivity. In the fluorescence experiments quenching was compensated for in a conceptually similar manner. After measuring the fluorescence in tissue samples, a known amount of AD 32 was added to each of the samples, and fluorescence was remeasured. For a given sample, the ratio of the expected increase in fluorescence (due to the addition of the AD 32 standard) to the observed increase was used as the quench correction factor for that tissue.

HPLC. Homogenates of small intestine (including contents) and liver were extracted in ethyl acetate/1-propanol and evaporated to dryness, as described above. Residues were redissolved in methanol. Aliquots of the methanol solution were analyzed on a Waters Associates ALC 244 liquid chromatograph equipped with a Waters Model 660 solvent programmer. Initially, anthracyclines were eluted from a μ-Bondapak/phenyl column (Waters Associates, Inc., Milford, MA) by means of solvent gradient of 26%–65% acetonitrile in pH 4.00 ammonium formate buffer run over 6 min (flow rate 2.0 ml/min). Later, however, the operating conditions were altered as needed, to facilitate preparative separations or to yield finer resolution. Wherever questionable, the identity of compounds was additionally confirmed by means of complementary normal phase LC analysis, according to conditions described previously [7, 8]. The specific conditions used are given in the legends to the figures. In all instances, signals were monitored by means of a Schoeffel Instrument Co. Model SF-970 flow fluorescence detector (excitation wavelength 482 nm, emission filter Schoeffel no. 2-73) interfaced with either a Hewlett-Packard Model 3380A recording integrator (reverse phase LC) or a Houston Omniscribe strip chart recorder (normal phase LC).

Extraction Efficiency. Known amounts of anthracycline standards were added to homogenates of liver and small intestine obtained from untreated mice. Recovery was determined by extracting and analyzing the compounds in a manner similar to that outlined above for tissues from AD 32-treated animals. In general, the recovery of N-trifluoroacetylated materials and aglycones was > 90%, whereas recovery for ADR and AMNOL averaged about 55% and 65%, respectively. Some conversion (< 5%) of AD 41 to its 13-dihydro-derivative, AD 92, was observed in liver and small intestine during the extraction process; all other standard compounds remained unaffected.

Isolation of Unknowns. Small amounts of purified unknowns for retention time analysis were obtained by HPLC by collecting the appropriate fractions. Larger amounts of material were obtained by pooling the liver homogenates from five to ten animals and extracting with ethyl acetate/1-propanol (9 : 1), as above. The organic extract was evaporated to dryness, and the residue was redissolved in chloroform and spotted as a streak on silica gel preparative TLC plates (1,000 µm thick layer; Analtech Inc., Newark, DE). TLC plates were developed with chloroform-methanol (9 : 1 by volume) and air-dried. The appropriate bands were scraped off and eluted with methanol. Preparative TLC was repeated until each unknown showed > 98% purity by HPLC.

Results

The levels of AD 32 and metabolites in various tissues 1 h after administration of AD 32, as determined either by radioactivity or by fluorescence, are shown in Table 1. In general, the levels of materials as determined by either method were approximately the same. Levels of radioactivity appeared to be slightly higher than fluorescence in pancreas, spleen, heart, and skeletal muscle. Levels of fluorescence were higher than the levels of radioactivity in the small intestine.

Of the tissues studied, highest levels of both fluorescence and radioactivity were found in the small intestine (including contents) and to a lesser extent in the liver. Intermediate levels of AD 32 and metabolites were seen in pancreas, kidney, spleen, large intestine, lung, and heart. Lower levels were found in muscle and fat. Low levels of radioactivity and anthracycline fluorescence were found in brain; these levels were only 5%–10% of that found in the viscera.

Table 1. Extractable metabolites from tissues of A/JAX mice 1 h after IV administration of a 50 mg/kg dose of AD 32

Tissue	Radioactivity		Fluorescence	
	Number of determinations[a]	Mean ± SEM nEq/g tissue	Number of determinations[a]	Mean ± SEM nEq/g tissue
Small intestine (including contents)	5	424.1 ± 24.1	6	508.8 ± 19.1
Liver	5	172.6 ± 18.3	6	208.3 ± 27.5
Pancreas	4	120.4 ± 9.3	6	86.8 ± 7.1
Kidney	5	98.3 ± 9.9	6	94.5 ± 6.1
Spleen	5	98.0 ± 3.3	4	57.0 ± 2.1
Large intestine (including contents)	3	95.4 ± 17.1	2	88.4
Lung	4	87.5 ± 10.0	6	73.4 ± 11.5
Heart	5	77.4 ± 5.0	5	61.2 ± 5.5
Muscle (skeletal)	4	44.7 ± 1.4	6	39.5 ± 1.6
Fat (omental)	4	25.9 ± 2.0	5	23.7 ± 1.5
Brain	5	6.6 ± 1.1	2	6.5

[a] Each determination represents a separate animal

HPLC analysis (flow fluorescence detection) of extracts of liver and small intestine, prepared from tissues of mice sacrificed 1 h after treatment with radiolabeled AD 32, revealed AD 41 (the predominant fluorescent species), AD 92, trace amounts of AD 32, and two new metabolites, designated unknowns 1 and 2.

Figure 1 shows the HPLC fluorescence trace of the separation of AD 41, AD 92, and the two unknowns from a sample of mouse liver homogenate extract on a phenyl/Corasil column (Waters Associates, Inc.). This column-packing material is capable of higher capacity (load), but has lower resolving power, than the μ-Bondapak/phenyl packing normally used for analytical separations in this laboratory. Superimposed on the fluorescence trace is the corresponding level of radioactivity, as determined by collecting fractions at 1-min intervals and monitoring these fractions by liquid scintillation counting. The initial peak of radioactivity, for which there was no comparable fluorescence signal, accounted for 40% of the injected radioactivity. No radioactivity was associated with the fluorescence signals for the two unknowns.

On the reversed phase μ-Bondapak/phenyl HPLC system, with the standard separation conditions described in *Materials and Methods*, unknown 1 and ADR showed similar retention times of about 2 min. The operating conditions were optimized to achieve better separation. When a sample of mouse liver

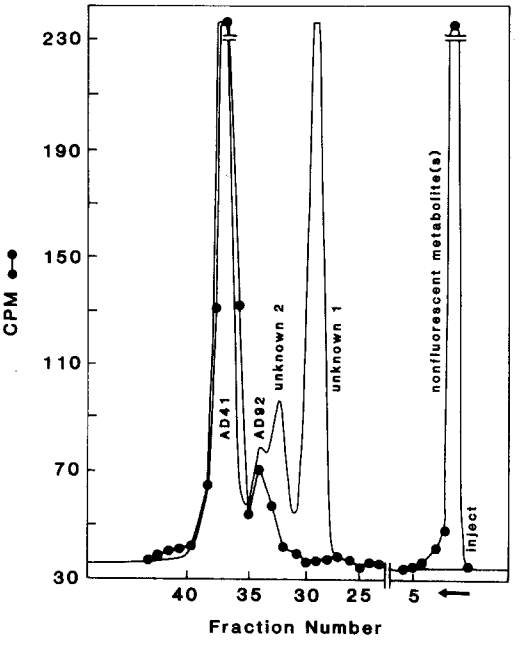

Fig. 1. Comparison of anthracycline fluorescence vs radioactivity in an extract of mouse liver homogenate at 1 h after administration of [^{14}C]-labeled AD 32 (50 mg/kg). Separation was achieved on a Waters Associates Model ALC 244 liquid chromatograph equipped with a 2 ft. × $^1/_8$ in. O.D. phenyl/Corasil column, with a linear elution program over 60 min of 0%–35% acetonitrile in aqueous ammonium formate buffer (0.1 *M*, pH 4.00) at a flow rate of 2.0 ml/min. Signal detection was by means of a Schoeffel Instrument Co. Model SF-970 spectrofluorometer. Fractions were collected at 1-min intervals for liquid scintillation counting and the counts were replotted over the fluorescence signal

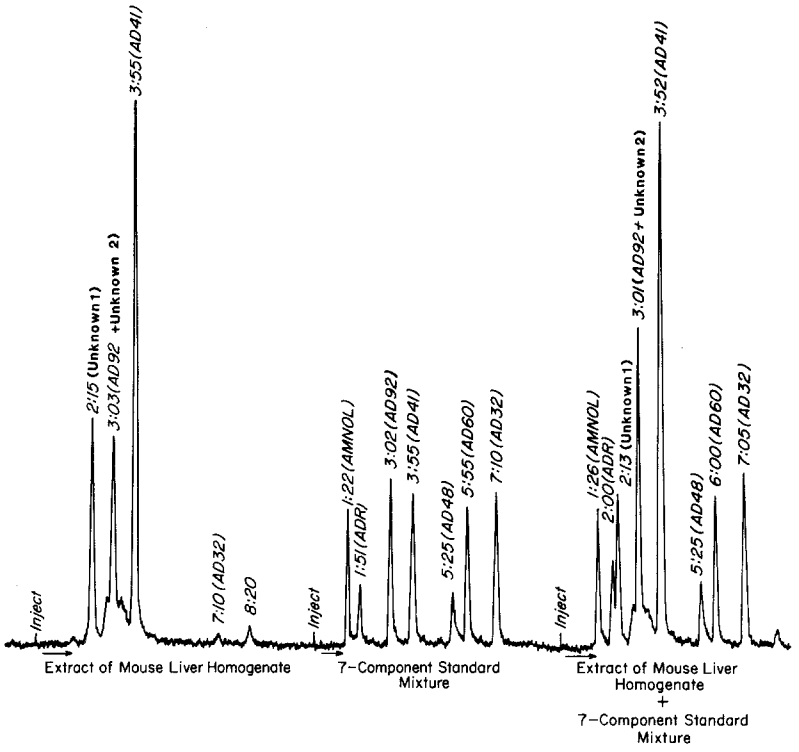

Fig. 2. Liquid chromatogram of a sample of extract of liver homogenate from a mouse treated with AD 32 (50 mg/kg IV) and sacrificed at 1 h (left-hand signal); a 7-component standard sample containing AMNOL, ADR, AD 92, AD 41, AD 48, AD 60, and AD 32 (center panel); and a mixture of the liver homogenate sample with the 7-component standard showing the lack of identity of unknown 1 with any of the known or putative metabolites of AD 32. Separations were achieved with a μ-Bondapak/phenyl reversed phase column and linear gradient elution for 7 min with 32%–65% acetonitrile in pH 4.00 ammonium formate buffer at 5 ml/min flow rate. Retention times are given in minutes: seconds

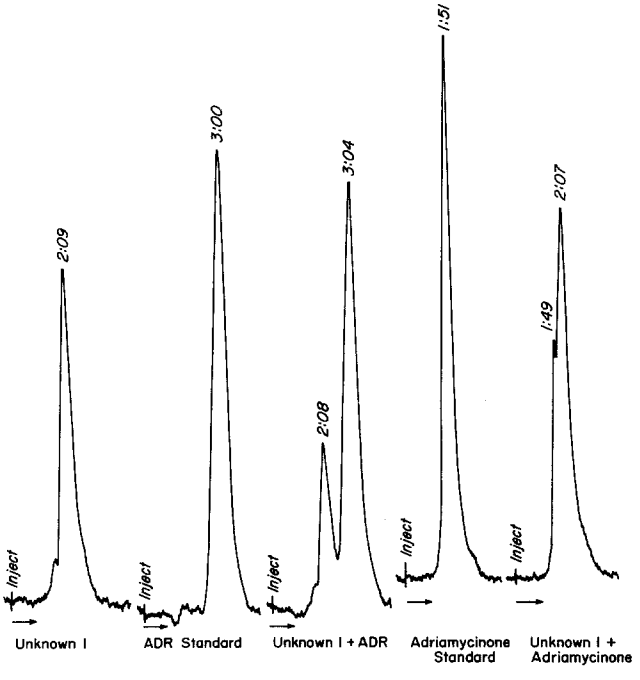

Fig. 3. HPLC signal of unknown 1, partially purified by TLC, and its lack of identity, according to retention time analysis, with ADR and the ADR aglycone, adriamycinone. Separations were accomplished on a Waters Associates Model ALC 202 liquid chromatograph, with a Whatman Partisil 10-PAC modified normal phase column (4.7 mm × 25 cm) and a developing system of chloroform-methanol-acetic acid-water (85 : 15 : 5 : 1.5 by volume) at 2 ml/min flow rate. Retention times are given in minutes : seconds

homogenate extract from a AD 32-treated animal was mixed with a 7-component standard mixture which included ADR, unknown 1 and ADR clearly separated from one another (Fig. 2). When run separately, however, unknown 1 and adriamycinone appeared to have the same retention times. To distinguish between these two compounds, the complementary normal phase HPLC system [7, 8] was used. As shown in Fig. 3, unknown 1 was different from both ADR and adriamycinone according to retention time analysis, and clearly resolved from each of these Standards when mixtures were injected.

Discussion

In the present study, the 1-h time point was selected for determination of tissue levels of radioactivity and total fluorescence, because previous work has indicated that by this time drug distribution is almost complete but elimination and cleavage of the radiolabel do not yet appear to be major events [9].

The high levels of fluorescence and radioactivity seen in the small intestine (including contents) and liver are consistent with the hepatobiliary pathway as the major route of excretion of AD 32 and metabolites, as has been established in rat [8], monkey [10], and man [2]. In the present study 37% of the administered radioactivity and 46% of the fluorescence were present in liver and small intestine at 1 h post drug administration.

N-Trifluoroacetylated anthracyclines are considerably more lipophilic in character than is ADR. The low levels of both radioactivity and fluorescence in brain, however, suggest that AD 32 and its major metabolites, AD 41 and AD 92, despite their lipophilicity, do not readily penetrate the blood-brain and blood-CSF barriers. This may explain the lack of antitumor activity of IV administered AD 32 against intracerebrally implanted L1210 leukemia in the mouse, as reported by Vecchi et al. [13].

As described earlier, a dose of radiolabeled AD 32 results in the persistence of radioactivity in mouse serum long after fluorescence has decayed. Furthermore, high levels of radioacitivity but little fluorescence are found in urine from these animals [9]. These results suggested that considerable cleavage of the radioactive label from the fluorescent chromophore had occurred. In part because of the extractability of the label from aqueous base into organic solvents, we believe the most likely nonfluorescent radioactive metabolite to be N-trifluoroacetyldaunosamine. Hydrolytic or reductive cleavage of the glycosidic moiety from AD 32, AD 41, or AD 92 would leave behind fluorescent aglycone species. A probable site for this cleavage reaction to occur might be liver and gut. HPLC examination of extracts of small intestine and liver homogenates did reveal two fluorescent metabolites of AD 32 not previously recognized in serum or bile. Small quantities of the unknown metabolites were obtained by HPLC or TLC separation of liver extracts, as described. The chromatographic properties of the two unknowns were unchanged on heating with dilute mineral acid, chemical conditions usually sufficient to hydrolyze anthracycline glycoside linkages. Thus, these materials appear to be aglycones, but neither matches adriamycinone (the ADR aglycone) (Fig. 3) or 13-dihydroadriamycinone (the AMNOL aglycone). Chemical chracterization is currently in progress.

Levels of metabolites in mouse tissues 1 h after treatment with AD 32, measured either by radioactivity or by fluorescence, exceeded the levels in the serum at the corresponding time. The increased level of AD 32 and metabolites in tissue compared with serum indicates that these compounds may be preferentially accumulated in tissues. A complete metabolism and disposition study has been initiated to examine this question.

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