Eur J Cancer Clin Oncol, Vol. 22, No. 4, pp. 451–460
Printed in Great Britain.

0277-5379/86$3.00+0.00

Disposition Kinetics of Adriamycin, Adriamycinol and Their 7-Deoxyaglycones in AKR Mice Bearing a Sub-Cutaneously Growing Ridgway Osteogenic Sarcoma (ROS)

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Abstract—*Disposition kinetics of Adriamycin (ADR), adriamycinol (AOL) and their 7-deoxyaglycones (ADR–DONE and AOL–DONE) have been studied in AKR mice bearing a s.c. growing ROS tumour after i.v. administration of* 10mg/kg. *ADR and its metabolites were extracted from tissues by two different methods, separated and identified by HPLC. Tissue 7-deoxyaglycones were isolated, purified and then identified by HPLC, TLC and mass spectrometry. Kinetic profiles of ADR showed rapid equilibration of the drug with well perfused tissues but a slower and complex equilibration of the drug with the ROS tumour. Serum and tissue profiles of AOL were similar to the parent drug. From the kinetic profiles of the 7-deoxyaglycones it appeared that in the tissues their formation was rapid, with ADR–DONE always appearing first. Maximum concentrations of ADR–DONE were reached in the liver and heart only* 10 min *after drug administration. Estimated half lives of ADR–DONE were in liver,* 1.1 hr *and in heart,* 2.8 hr *and for AOL–DONE in liver,* 5.4 hr, *in heart,* 5.1 hr *and in serum,* 4.1 hr.

INTRODUCTION

A UNIQUE feature of the *in vitro* biotransformation of Adriamycin (ADR), which only occurs under anaerobic conditions, is the formation of 7-deoxyaglycone metabolites by reductive removal of the C-7 linked daunosamine sugar group (ADR–DONE and AOL–DONE, *5* and *6*, Fig. 1 [1]). ADR, and its C-13 carbonyl reduction product adriamycinol (AOL, *2*, Fig. 1) and their 7-hydroxyaglycones (ADR–ONE and AOL–ONE, *3* and *4*, Fig. 1) can all act as substrates for the large variety of enzymes which can catalyse this 'glycosidase' reaction [2–4]. In particular, ADR has an affinity for one electron transporting flavoenzymes and this has been attributed to the formation of a complex between the drug and the co-enzyme nucleotides FAD and FMN [5]. Under aerobic conditions, *in vitro*, ADR is preferentially reduced by one electron transporting flavoenzymes to a semi-quinone free radical intermediate, which has the property of being able to continuously transport reducing equivalents to another donor molecule (in most incubations molecular oxygen) without itself being altered structurally [6, 7]. The deleterious effects of large scale production of reactive oxygen species on cells, *in vivo*, for example lipid peroxidation, has been strongly implicated in ADR induced toxicity [8, 9].

The exact mechanism of anaerobic formation of a 7-deoxyaglycone metabolite of ADR is not known but it is likely that it is a complex reaction which can proceed either through a one or two electron reduction pathway (Fig. 1a, [10]). The first stage in the one electron reduction pathway is formation of the semi-quinone intermediate, but it being unable to transfer its free electron to oxygen rearranges to a lower energy form by transferring the radical centre to position C-7 and eliminating the daunosamine sugar group (Fig. 1a). Protonation of the C-7 aglycone radical may terminate the sequence and yield the 7-deoxyaglycone [11]. The C-7 radical is detected during enzymatic reduction of ADR to a 7-deoxyglycone by NADPH-cytochrome c reductase [12]. A similar molecular rearrangement is believed to occur when aclacinomycin is reduced anaerobically to the aglycone dimer 7,7'-

Accepted 15 October 1985.
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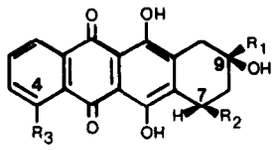
Work supported with grant from The Cancer Research Campaign, London, U.K. (CRC SP1429 P3)



COMPOUND	R_1	R_2	R_3
1 ADR	$-C(=O)-CH_2OH$ (13)	daunosamine	OCH_3
2 AOL	$-CH(OH)-CH_2OH$	daunosamine	OCH_3
3 ADR-ONE	$-C(=O)-CH_2OH$	OH	OCH_3
4 AOL-ONE	$-CH(OH)-CH_2OH$	OH	OCH_3
5 ADR-DONE	$-C(=O)-CH_2OH$	H	OCH_3
6 AOL-DONE	$-CH(OH)-CH_2OH$	H	OCH_3

Fig. 1. *The structure of Adriamycin and its metabolites.*

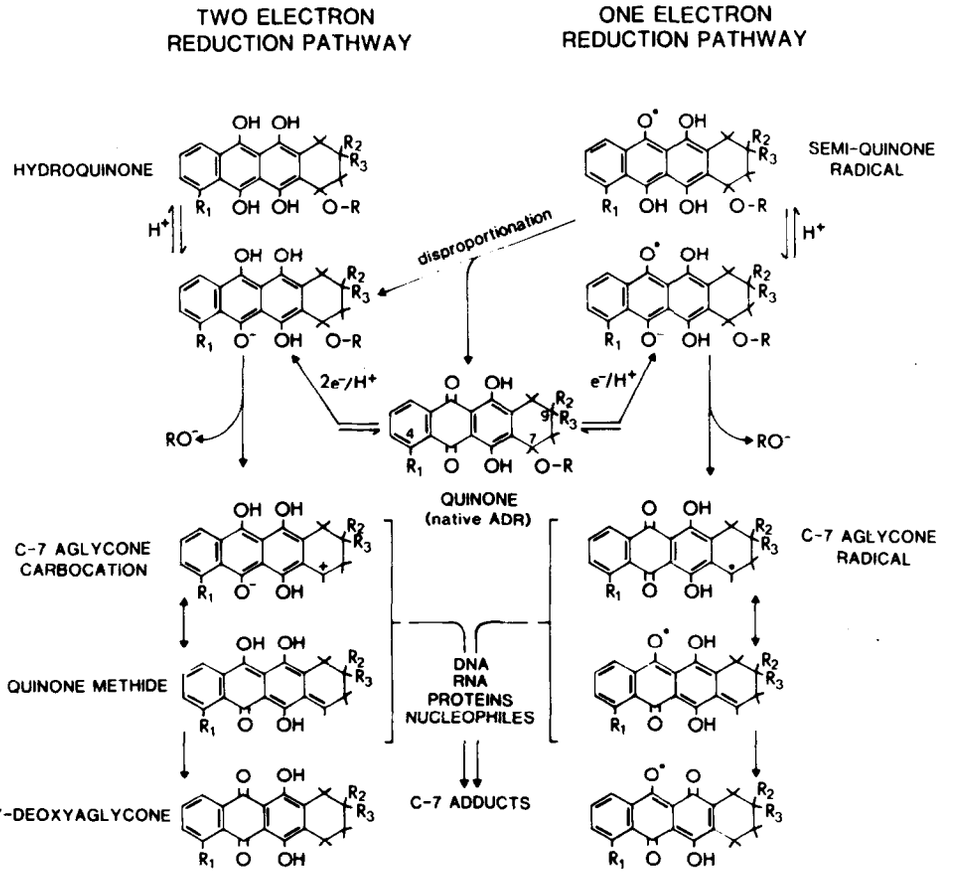


Fig. 1a. *Proposed pathways of metabolic activation of Adriamycin to long-lived reactive DNA alkylating agents by anaerobic one and two electron reduction mechanisms (taken from a figure in reference* [16]).

bis (7-deoxyaklavinone) [13]. The two electron reduction pathway envisages the 7-deoxyaglycone formed from long lived reactive intermediates, other than the semi-quinone, which can diffuse through the cell and alkylate DNA [14]. These intermediates may be involved with anthracyclines which do not intercalate with DNA but which, nevertheless, induce DNA strand scission and DNA/protein interactions [15]. Two electron reduction of ADR produces a hydroquinone intermediate that on ionisation can eliminate daunosamine to leave a C-7 carbocation aglycone which due to full resonance with a quinone methide aglycone is sufficiently long lived to fulfil the criteria of an active alkylating agent [16] (Fig. 1a). The 7-deoxyaglycone is derived from quinone methide by intramolecular proton transposition [16].

7-Deoxyaglycone metabolites of ADR have been identified in the urine of cancer patients providing some evidence that formation of these reactive intermediates may also occur in man [17]. We have recently identified 7-deoxyaglycones in the serum of patients [18] and in this report we have attempted to study the pattern of their tissue disposition, *in vivo*, in tumour-bearing animals. The mouse was chosen as the model because its tissue anaerobic glycosidase activities were shown to be almost identical to man *in vitro* [4].

MATERIALS AND METHODS

Chemicals and reference compounds

All solvents used for HPLC and all methanol, chloroform and propan-2-ol used were of HPLC reagent grade (Fisons Scientific Apparatus, Loughborough, U.K.). Water was de-ionised and double distilled in a quartz glass still. Pure Adriamycin–HCl and adriamycinol–HCl were a gift from Dr. S. Penco (Farmitalia, Milan, Italy) and daunorubicin–HCl (DNR) was from May and Baker Ltd. (Dagenham, U.K.). Four different aglycone metabolites were used as reference compounds for the HPLC assay. Adriamycin aglycone and Adriamycin 7-deoxyaglycone were from Dr. Penco and were also synthesised. ADR–ONE and AOL–ONE were synthesised by mild acid hydrolysis with 0.1M HCl at 55°C for 1 hr [19] and ADR–DONE and AOL–DONE were synthesised by catalytic hydrogenation using a palladium catalyst [18]. Purity of all the synthesised aglycone standards has already been reported [18].

Animals

All the animals used were from an inbred colony of AKR mice. The ROS tumour was kindly supplied by Dr. F.M. Schabel Jr. (Southern Research Institute, Alabama, U.S.A.). It was maintained in the Oncology Department by serial sub-cutaneous passage of 100 mg tumour fragments through female AKR mice. Tumour weight was estimated by caliper measurements and mice bearing tumours of 1.0–1.5 g were used in all the experiments. The sensitivity of the ROS to ADR has been reported elsewhere [20]. Animals were housed in plastic cages in conditions of heating, lighting and feeding which were kept constant throughout.

Sampling

Mice were killed with an overdose of ether at 5 min, 10 min, 20 min, 30 min, 45 min, 60 min, 90 min, 2 hr, 4 hr, 6 hr, 8 hr and 24 hr after i.v. administration of 10mg/kg Adriamycin–HCl. Drug was delivered to animals anaesthetised with halothane via a tail vein. Preliminary studies showed that the initial period of anaesthesia did not alter metabolite profiles in liver, heart, kidney or tumour. At each time an aliquot of blood (0.8 ml approx.) was removed from the vena cava with a syringe and hypodermic needle; the whole liver, heart and tumour were collected and washed with buffered sterile 0.9% NaCl (pH 7.4) before being immediately frozen to −60°C with solid CO_2. Blood was allowed to clot then serum was separated by centrifugation and stored at −20°C. Tissue was also stored at −20°C. Prior to extraction heart was chopped into small pieces and washed again in 0.9% NaCl to further remove blood. Neither blood nor tissues from individual animals were pooled. Four animals were killed at each time for four separate determinations of drug and metabolite concentrations.

High performance liquid chromatography

The HPLC method used in this work has already been described in detail [18]. Although the method was originally developed to analyse human serum, retention times (t_R) and column capacity factors (k') of ADR and metabolites extracted from untreated tissue homogenates were identical to t_R and k' of standard reference compounds. Without loss of resolution t_R could be modified by changing the proportions of the constituents of the mobile phase.

Serum extraction

Rapid extraction from serum was performed as previously described [18]. Essentially, the method was the same as tissue extraction method 1 (see below).

Tissue extraction: method 1, *direct extraction*

Prior to extraction sera and tissues were allowed to thaw at room temperature. One to two grams of liver, heart and tumour were suspended in 3 vol of buffered 0.9% NaCl and finely minced with an

Ultra Turrex electrically driven rotating cutting blade for 30 sec. A homogenate was produced with five up and down strokes with a Potter Elvehjem homogeniser. Light microscopy confirmed that cells were disrupted by this procedure. To 1 ml of homogenate was added DNR as an internal standard. Drug and metabolites were extracted by adding 5 vol of chloroform/propan-2-ol (2 : 1) and vortexing for 30 min in a Buchler vortex evaporator (supplied by Gallenkemp, East Kilbride, U.K.). Centrifugation at 1000 ***g*** for 15 min separated three phases: an upper aqueous phase, a middle tissue pellet and a lower organic phase. The upper aqueous phase was discarded by aspiration and the lower organic phase was decanted over the tissue pellet and transferred to a clean test tube to be evaporated to dryness in the vortex evaporator at 40°C and 25mm Hg of vacuum. The dry extracts were reconstituted in a small volume of methanol and were ready for HPLC separation.

Tissue extraction: method 2, pretreatment of tissue homogenates with silver ions

Method 2 was modified from the extraction method of Schwartz [21]. Tissue was homogenised as in the previous extraction method and DNR was again the internal standard. One ml of tissue homogenate was treated with 33% (w/v) silver nitrate (0.2 ml per ml homogenate) for 10 min at 4°C in the vortex evaporator with vigorous shaking. The vigorous shaking was important for a good recovery. Immediately, 5 vol. of chloroform/propan-2-ol (2 : 1) was added to the homogenate mixture and ADR and its metabolites were extracted by vortexing for a further 30 min. Three phases were separated by centrifugation at 1000 ***g*** for 15 min. The upper aqueous phase was discarded by aspiration, the lower organic phase was decanted over the middle tissue pellet into a clean test tube and evaporated to dryness in the vortex evaporator as before. The samples were then ready for HPLC.

Mathematical analysis

The area under the curve (AUC_{0-24hr}) was calculated by the trapezoidal rule. Pharmacokinetic parameters were calculated from an extended least squares computer fit to the experimentally determined serum concentrations of ADR.

Mass spectrometry

A mass spectrum of methanolic solutions of AOL–DONE isolated from liver, heart and serum extracts during HPLC using a Gilson Microcol TDC 80 fraction collector (Gilson, Villiers-le-Bel, France) was obtained by direct probe injection mass spectrometry using a Kratos MS 902S mass spectrometer and a DS 55C data system (Kratos Analytical Instruments, Urminston, U.K.) set at 500 μA and 70 eV. Source temperature was set at 220°C.

RESULTS

In control experiments liver, heart and ROS tumour homogenates were spiked with 20 ng or 2 μg of ADR, AOL, AOL–DONE and ADR–DONE and extracted using the two methods described. Recoveries ranged from 68 to 91%, were independent of the concentration and the four compounds were extracted with similar efficiency. Also, there were no statistically significant differences in recovery when the two methods were compared. Both methods did not extract endogenous compounds which interfered with the identification of ADR and its metabolites by HPLC. Method 1 extracted only one endogenous fluorescent peak (peak 1, see Fig. 3) and it always eluted before ADR and its metabolites, close to the solvent front. Method 2 did not extract this peak or any other fluorescent endogenous peak (not shown).

Metabolite identification by high performance liquid chromatography

Serum and tissue metabolites were identified by comparing their k' values with k' values of chemically pure reference metabolites of ADR. Figure 2 shows a chromatographic separation of ADR and all the reference metabolites used in this work and

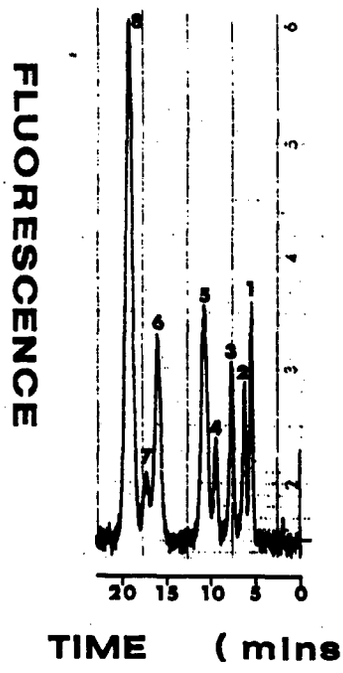


Fig. 2. Separation of reference standards of Adriamycin and its metabolites by HPLC. Peaks are identified as: 1, *AOL*; 2, *AOL–ONE*; 3, *ADR*; 4, *ADR–ONE*; 5, *AOL–DONE*; 6, *DNR*; 7, *ADR–DONE and* 8, *DNR aglycone. Chromatographic conditions were: stationary phase*, μ-*Bondapak* C18 (*column dimensions* 25 cm × 4.6 mm I.D.); *mobile phase*, 5mM (*final concentration*) *phosphoric acid*, 62.5% *in methanol: acetonitrile: propan-2-ol* (12.5%:12.5%:12.5%), pH 3.2 *and flow rate* 2.5 ml/min. *(Reproduced by kind permission of Elsevier Scientific Publishing Company, Amsterdam from* [18].)

in a series of chromatograms Fig. 3 shows the metabolite species which were extracted and separated from various tissues, the ROS tumour and serum of AKR mice. In each chromatogram of Fig. 3 chromatographic conditions were optimised to best resolve all the species detected and are described in the legend to Fig. 3. In the liver five metabolites were detected, three co-eluted with the reference metabolites of AOL, AOL–DONE and ADR–DONE and the other two remain unidentified. The first of the unidentified metabolites eluted near ADR ($k' = 6$, Fig. 3) and is believed to be an intact glycoside, the second eluted close to ADR–DONE ($k' = 20$) and is believed to be an aglycone. All the peaks separated from all other tissues, the ROS tumour and serum co-chromatographed with reference metabolites: in the kidney these were, AOL, AOL–DONE and ADR–DONE; in the heart, AOL–DONE and ADR–DONE; in the ROS tumour, AOL–DONE and in serum, AOL and AOL–DONE (Fig. 3).

Identification of the 7-deoxyaglycone metabolites

The chromatographic peaks corresponding to AOL–DONE and ADR–DONE were collected during several HPLC analyses of liver, heart and serum extracts and concentrated. Both compounds were re-chromatographed using TLC and three different ascending solvent systems (techniques described in [18]). The isolated metabolites chromatographed as single spots implying purity even though they were collected from different tissues and serum, with R_f (retardation factors) values identical to the reference compounds of AOL–DONE and ADR–DONE. Direct probe mass spectrometry of isolated AOL–DONE confirmed the identity of the metabolite. Its spectrum showed that the most abundant ion (100%) was the parent m/z, mass 400 corresponding to the molecular weight of AOL–DONE. The most abundant fragment (96%) was m/z 339 which corresponds to a recognisable fragment of AOL–DONE (M–CHOH and CH_2OH).

Comparative tissue contents of Adriamycin and metabolites

A total tissue content was calculated by integrating the area under concentration/time profiles from 0 time to 24 hr (AUC). Results for ADR, AOL, AOL–DONE and ADR–DONE in liver, heart, tumour and serum are in Table 1. In the liver

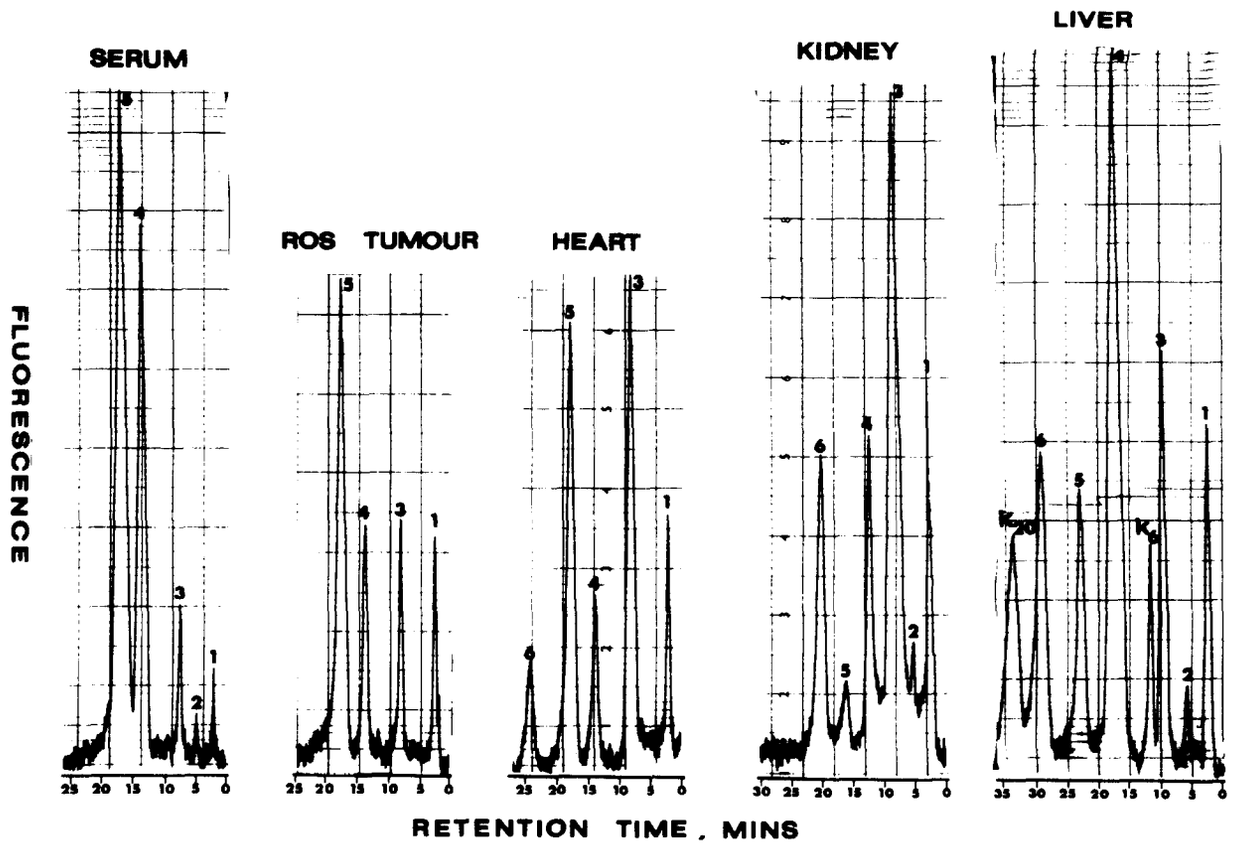


Fig. 3. Identification of Adriamycin and its metabolites in AKR mice bearing a s.c. ROS tumour after administration of 10 mg/kg *i.v. Each chromatogram represents a sample taken* 20 min *after drug administration. Peaks are identified as:* 1, *endogenous;* 2, *AOL;* 3, *ADR;* 4, *AOL–DONE;* 5, *DNR (internal standard) and* 6, *ADR–DONE. In the liver two peaks did not elute against known standards and were ascribed names according to their column capacity factors* (k'). *Chromatographic conditions were: stationary phase as in Figure* 2; *mobile phase,* 5mM *(final concentration) phosphoric acid,* 62.5% *in methanol:acetonitrile:propan-2-ol,* 18%:18%:1.5% *for liver,* 12.5%:12.5%:12.5% *for kidney,* 16.5%:16.5%:4.5% *for heart, ROS tumour and serum,* pH 3.2 *and flow rate* 2.5 ml/min.

Table 1. Comparative total tissue concentrations of Adriamycin (ADR), adriamycinol (AOL), adriamycinol 7-deoxyaglycone (AOL-DONE) and Adriamycin 7-deoxyaglycone (ADR-DONE) expressed as the AUC_{0-24hr} of their serum and tissue concentration/time profiles

	*ADR		AOL		AOL-DONE		ADR-DONE	
	†1	†2	1	2	1	2	1	2
Liver	21.9	30.9	1.8	7.6	10.3	13.7	5.3	5.2
Heart	36.5	38.4	0.0	0.0	1.1	1.3	0.6	0.7
Tumour	1.6	11.4	0.0	0.0	0.1	0.1	0.0	0.0
Serum	2.3	–	0.04		1.4	–	0.0	–

* Tissue AUC, μg/g tissue × hr, serum AUC, μg/ml serum × hr.

† Results obtained using different tissue extraction techniques:
1, extraction method 1 (direct extraction of homogenates with chloroform/propan-2-ol (2:1)); 2, extraction method 2 (pretreatment of homogenates with 33% (w/v) silver nitrate before extraction with organic solvent, see Materials and Methods).

where additional unidentified metabolites of ADR were detected $k'6$ was quantitated assuming molar fluorescence equal to ADR and $k'20$ assuming molar fluorescence equal to ADR–DONE. Their AUCs were 0.4 ($k'6$) and1.3 ($k'20$) μg/g × hr, which accounted for 3% of the total AUC of all the ADR species detected in the liver.

Total concentration of the drug and its metabolites extracted by method 2 was always slightly higher than by method 1 with two apparent exceptions: AOL in the liver and the parent drug in the ROS tumour (Table 1). Here, method 2 recovered several times more than method 1. Total content of ADR and metabolites identified in the liver was 59.1 μg/g × hr of which 52% was ADR, 23% AOL–DONE, 12% AOL and 9% ADR–DONE; in the heart 40.4 μg/g × hr of which 95% was ADR, 3% AOL–DONE and 2% ADR–DONE and in the ROS tumour 11.5 μg/g × hr of which 99% was ADR and 1% AOL–DONE (Table 1).

Kinetic profiles of Adriamycin and its metabolites in serum, liver, heart and ROS tumour of AKR mice after 10 *mg/kg i.v.*

The serum (Fig. 4), liver (Fig. 5) and heart (Fig. 6) profiles refer to the results obtained with tissue extraction method 2. The results obtained with tissue extraction method 1 were similar, although the levels were always slightly lower, and yielded identical half lives. Only with the ROS tumour did the profiles differ significantly when the two different extraction methods were used and both are shown in Fig. 7. Each point in Figs. 4–7 represents the mean value of four separate determinations. Coefficients of variation in all the mean values ranged from 6 to 21%.

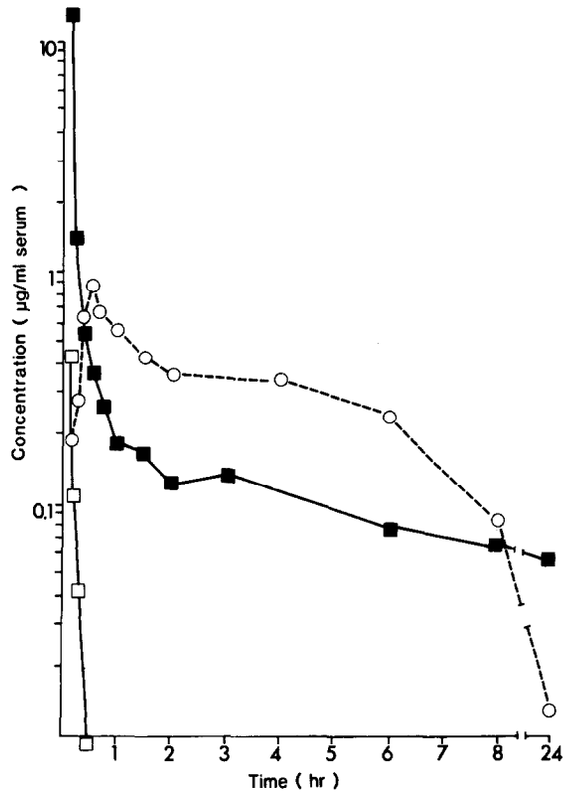


Fig. 4. Concentration/time profiles of Adriamycin and its metabolites in the serum of AKR mice bearing a s.c. ROS tumour after administration of 10 mg/kg *i.v.* ■——■, *ADR;* ○----○, *AOL–DONE and* □——□, *AOL. Each point represents the mean of four separate determinations with coefficients of variation ranging from* 6 *to* 21%.

Adriamycin

The serum decay profile of ADR (Fig. 4) was best fitted by the tri-exponential expression: serum concentration (c) = $164e^{-31.5t}$ + $0.83e^{-2.29t}$ + $0.12e^{-0.038t}$, where the constants 164, 0.83 and 0.12 are in μg units. The $t_{\frac{1}{2}}\alpha$ was 1.3 min, indicating rapid initial distribution: $t_{\frac{1}{2}}\beta$, imagined as representing distribution into a less well perfused body compartment and or a metabolic process was 18.2 min and the $t_{\frac{1}{2}}\gamma$, the half life of the drug was 18.2 hr.

Rapid equilibration of ADR with both the liver (Fig. 5) and the heart (Fig. 6) was achieved only 10 min after drug administration. Equilibration of ADR with the ROS tumour was biphasic (Fig. 7).

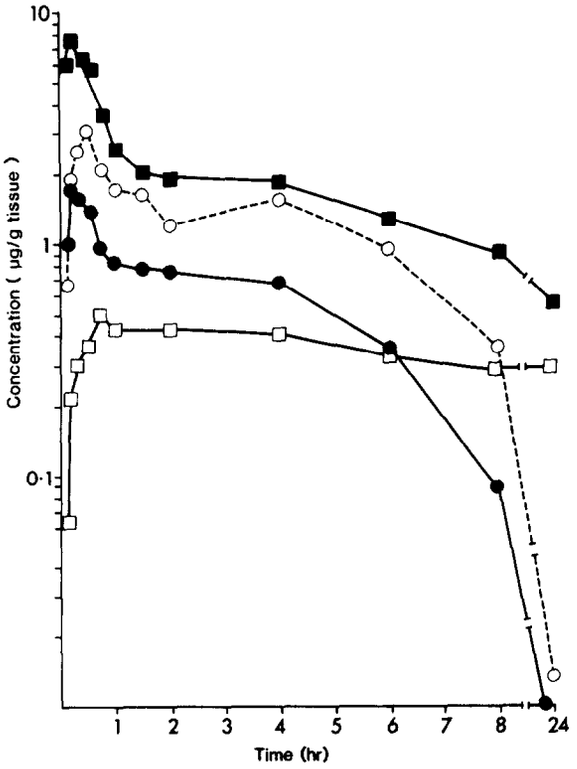


Fig. 5. *Concentration/time profiles of Adriamycin and its metabolites in the liver of AKR mice bearing a s.c. ROS tumour after administration of* 10 mg/kg *i.v.* ■——■, *ADR*; ○----○, *AOL-DONE*; ●——●, *ADR-DONE and* □——□, *AOL. Each points represents the mean of four separate determinations with coefficients of variation ranging from* 6 *to* 21%.

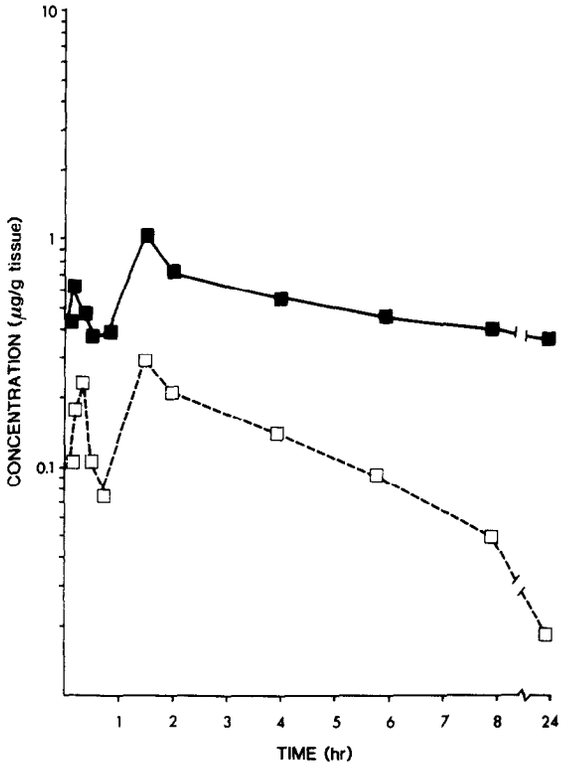


Fig. 7. Concentration/time profile of Adriamycin in the s.c. ROS tumour after administration of 10 mg/kg *i.v.* ■——■, *Results obtained using tissue extraction method* 2 (*see Materials and Methods*). □---□, *Results obtained using tissue extraction method* 1. *Each point represents the mean of four separate determinations with coefficients of variation ranging from* 6 *to* 21%.

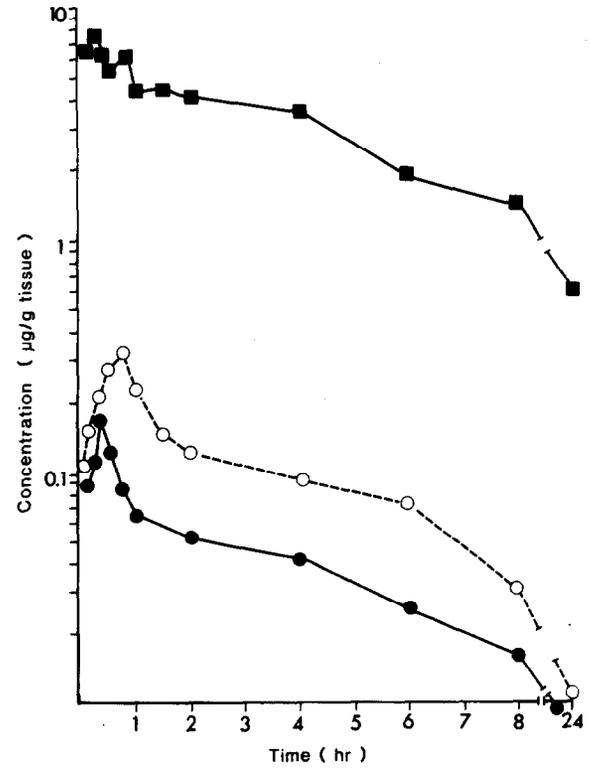


Fig. 6. Concentration/time profiles of Adriamycin and its metabolites in the heart of AKR mice bearing a s.c. ROS tumour after administration of 10 mg/kg *i.v.* ■——■, *ADR*; ○----○, *AOL-DONE and* ●——●, *ADR-DONE. Each point represents the mean of four separate determinations with coefficients of variation ranging from* 6 *to* 21%.

According to method 1 the first phase reached equilibration with serum after 20 min and the second phase reached equilibration after 90 min. According to method 2 the first phase reached equilibration with serum after only 10 min and the second phase reached equilibration with serum after 90 min. From the linear terminal phase of ADR tissue profiles a half life was calculated by non-linear regression data fitting. The half life in the liver was estimated to be 13 hr, in the heart 9 hr and in the ROS tumour 7 hr according to extraction method 1 and 30 hr according to extraction method 2.

Adriamycinol

AOL was detected in serum for only 20 min during which time its concentration declined similarly to the α phase of ADR's serum profile and with an apparent half life equivalent to 2 min (Fig. 4). In the liver it reached a maximum concentration of 480 ng/g tissue by 45 min after which time its level remained virtually constant (Fig. 5) with a half life greater than 50 hr.

Adriamycin 7-deoxyaglycone

The profiles of ADR–DONE in the liver and heart showed a steep rise in concentration to early peak levels coinciding with parent drug equilibra-

tion in the liver (Fig. 5) and shortly after parent drug equilibration in the heart (Fig. 6). Calculated from the terminal phase of the tissue profiles (Figs. 5 and 6) the half life of ADR–DONE was estimated to be 1.1 hr in the liver and 2.8 hr in the heart.

Adriamycinol 7-deoxyaglycone

The tissue profiles of AOL–DONE were also characterised by an initial steep rise in concentration, always following close after the appearance of ADR–DONE but achieving greater maximum levels (Figs. 5 and 6). Estimated half lives were 5.4 hr in the liver, 5.1 hr in the heart and 4.1 hr in serum. The serum profile of AOL–DONE was similar to its tissue profiles and between 20 min and 8 hr its serum concentrations were greater than the serum concentrations of ADR (Fig. 4). A secondary peak concentration of AOL–DONE was observed in the liver profile at 4 hr (Fig. 5).

DISCUSSION

Cytofluorescence microscopy and sub-cellular fractionation studies have shown that ADR almost entirely localises in the nucleus of cells [22–24]. Furthermore, the concentration of ADR found in animal tissues after i.v. administration correlated with the number of nuclei present in a particular tissue on a gram for gram basis [24, 25]. It appears that the nucleus of the cell can both influence the intracellular distribution and *in vivo* tissue distribution of the drug. One possible explanation why it can is that ADR has a high affinity for binding to nucleic acids either by intercalation with the double helix or through covalent or ionic linkage [26] resulting in it becoming physically immobilised within the nucleus [27]. Efflux of an ADR analogue from cultured human CCRF–CEM cells followed first order kinetics with a terminal phase half life of 20–74 hr [28] indicating that ADR is probably only slowly released from its binding sites within the nucleus. The human pharmacokinetics of ADR are also characterised by a long terminal phase half life of the same order as the slow efflux of drug anthracyclines from cells [29] suggesting that its elimination in man may also be governed by slow release of nuclear bound drug from the tissues.

In AKR mice we have shown that the elimination phase calculated from serum kinetics ($t_{\frac{1}{2}}$ 18.2 hr) reflects relatively long retention of the drug in the tissues: for example liver $t_{\frac{1}{2}}$ 13 hr; heart $t_{\frac{1}{2}}$ 9 hr and the ROS tumour $t_{\frac{1}{2}}$ 30 hr. The value of the half life for the tumour is seen to represent either a greater degree or higher affinity of intracellular binding. This conclusion may also be implied from the results obtained using two different tissue extraction techniques. Extraction method 1 is a non-destructive technique which extracts ADR and its metabolites from homogenates directly with an excess of an organic solvent mixture. Extraction method 2 differs from 1 in that homogenates are pretreated with silver ions before organic solvent extraction. Silver nitrate precipitates proteins but also interacts with nucleotides releasing ADR (and presumably its metabolites) which are intercalated with the double helix of DNA and RNA [21]. Non-destructive, direct tissue extraction techniques like method 1 recovered only a fraction of DNR present in rat liver tissue slices and have been criticised due to their inability to release bound anthracyclines [30–32]. We used such a method to estimate what we believe is a freely extractable fraction of ADR and metabolites in tissues and pretreatment with silver ions to release drug reversibly complexed with cellular macromolecules, especially nucleic acids. Thus, an increase in recovery due to method 2 should indicate drug being released from intracellular binding sites. Pretreatment of homogenates with silver ions increased the yield of ADR from the ROS tumour by 7-fold compared to direct extraction whilst only improving yield from the liver by 40% and not improving yield from the heart. Also, ADR extracted from the tumour by method 2 had a half life 4 times longer than ADR extracted by method 1. Both these results, the increased yield and longer half life, we interpret to reflect the greater binding of ADR in the ROS tumour.

Kinetic profiles of the C-13 carbonyl reduced glycosidic metabolite AOL are also consistent with a high affinity/slow release binding mechanism. These data include: rapid disappearance from serum; prolonged retention in the liver with a half life of greater than 50 hr and 4-fold increase in yield from liver after silver pretreatment.

Schwartz [33] has proposed that the aglycone metabolites of ADR may also be retained in the tissues, trapped in lipid membranes, not because of high affinity binding but due to their relatively greater lipophilicity than the parent glycosides. The tissue profiles of two aglycones ADR–DONE and AOL–DONE show that they are not retained to the same degree as their parent glycosides ADR and AOL. It has been shown that alterations in the daunosamine sugar group of anthracyclines greatly reduces their association with DNA (34) highlighting the importance of the sugar moiety for binding.

Under anaerobic conditions, *in vitro*, enzymatic reductive glycosidic cleavage of ADR to 7-deoxyaglycones proceeds at high rates of catalysis [4]. However, because 7-deoxyaglycone metabolites have been rarely detected in substantial concentrations, in the tissues of a number of animal species [35–37], it has been concluded that glycosidase activity is not normally expressed *in vivo* [4]. In tumour bearing AKR mice we have demonstrated evidence of significant tissue glycosidase activity operating *in vivo*.

Formation of 7-deoxyaglycone metabolites of DNR by anaerobic rat liver microsomes was shown to proceed through a linear sequential pathway and obey first order kinetics: DNR to DNR 7-deoxyaglycone (DNR–DONE), DNR–DONE to daunorubicinol 7-deoxyaglycone [38]. Later, a similar pathway was reported for ADR biotransformation by anaerobic rat liver microsomes [39]. Anaerobic rat liver microsomes converted as much as 80% of ADR to ADR–DONE within the first 2 min of incubation [40]. Thenafter, the concentration of ADR–DONE fell to a relatively low level as the concentration of AOL–DONE increased to a maximum value by 30 min. The kinetic profiles of ADR–DONE and AOL–DONE, although complex, are consistent with the above pathway of formation occurring in the tissues of tumour bearing AKR mice *in vivo*. Liver and heart concentrations of ADR–DONE increased almost simultaneously with the influx of ADR into these tissues and then fell sharply, presumably due to both immobilisation of the parent drug by binding and utilisation of the aglycone as the substrate for AOL–DONE formation. Changes in AOL–DONE concentrations always followed shortly after changes in ADR–DONE concentrations but higher levels of the former were always attained. In fact, conversion of ADR–DONE to AOL–DONE in the tissues may proceed to completion as ADR–DONE was not detected in serum. In the ROS tumour, where only AOL–DONE was detected, we believe that the aglycone could not have been produced locally but was probably deposited there by the circulation. Our own *in vitro* studies (unpublished observations) support this view. Here anaerobic AKR mouse liver and heart microsomal and mitochondrial fractions converted ADR to 7-deoxyaglycones in the presence of NADPH and NADH but microsomal and mitochondrial fractions prepared from the ROS tumour did not.

In conclusion: 7-deoxyaglycones are important metabolites of ADR because they are the end products of biotransformations that involve reactive intermediates which can participate in both the anticancer action and toxicity of the drug. We have demonstrated evidence that 7-deoxyaglycones are formed in animal tissues *in vivo* and described their kinetics. Finally, the AKR mouse would appear to be a good model to further study factors which affect 7-deoxyaglycone production in tissues.

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