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Comparative Metabolism and Excretion of Adriamycin in Man, Monkey, and Rat

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Summary. *Adriamycin and its fluorescent metabolites in bile (man, monkey, and rat) and urine (man and monkey) were determined by means of a simple, rapid, and highly reproducible high-performance liquid chromatographic procedure. Species differences in metabolism and biliary excretion were observed with respect to aldo-keto reductase and conjugase activities.*

Introduction

Adriamycin (ADR) is an anthracycline glycoside antibiotic now widely used in the treatment of malignant disease [6]. In man, following IV administration, there is rapid distribution of drug throughout the tissues [5, 22] and then slow elimination, principally by excretion in the bile [9, 18]. Biliary excretion is also the major excretory pathway in several animal species, but there is some dispute regarding the extent of metabolism and the number of metabolites formed [4, 17, 20, 23]. A small proportion of the administered dose can also be accounted for in the urine, but again there is disagreement regarding the number of metabolites present, the number reported ranging from none [19] to a high number including aglycones and conjugates [21]. A possible explanation for these disparate results relates to the use of an analytical methodology [thin layer chromatography (TLC)] that has been shown [13, 14] to give rise to artifactual products.

In connection with mechanism of action studies with a highly promising ADR analog [12] under development in these laboratories, we have had to explore alternative methods for the identification and quantitation of fluorescent anthracyclines. This has led to the development of an unambiguous and nondestructive assay of ADR and 13-dihydroadriamycin (adriamycinol, AMNOL) by high-performance liquid chromatography (HPLC), with fluorescence detection capable of estimating ADR and AMNOL at a lower limit of sensitivity of 2–5 pmol [8, 9, 13–15]. We report here our findings on the biliary and urinary excretion and metabolism of ADR in man, monkey, and rat, as determined by this method.

Materials and Methods

Drugs. Commercial ADR hydrochloride formulated with lactose for IV administration in saline was used for clinical treatment. For investigational use, bulk ADR hydrochloride, lot no. 64498, kindly provided by Farmitalia S.p.A., Milan, Italy, was used in Emulphor (polyethoxylated castor oil)-ethanol-isotonic saline, 5 : 5 : 90 by volume (2 mg/ml). Emulphor-ethanol, as a 1 : 1 concentrate, was kindly provided by Mr. J. Paul Davignon, Pharmaceutical Resources Branch, Division of Cancer Treatment, National Cancer Institute.

AMNOL, adriamycinone, and 13-dihydroadriamycinone, used as reference standards for the quantitation and identification of possible metabolites, were prepared in this laboratory. The authenticity of each was confirmed by microchemical analysis, and each compound was more than 99% pure, as determined by HPLC.

Patients. Serial bile samples were obtained from two patients with indwelling T-tubes. Both had disseminated disease and were to receive ADR as planned treatment. Liver function, as judged by routine biochemical and radioisotopic screening, was normal. The patients were under the care of the staff of the Medical Oncology Division at the Sidney Farber Cancer Institute, and informed consent was obtained in the usual manner. Biliary obstruction was complete in one patient, as judged by radiologic examination. ADR (60 mg/m^2) was administered as a single IV bolus.

Animals. One conditioned female cynomolgus monkey (*Macaca fascicularis*) weighing 2.75 kg was obtained from Hazelton Prime Labs., Mount Clair, NJ, and was observed for a 2-week holding period. On the day of study, the animal was premedicated with ketamine hydrochloride (15 mg/kg), and anesthesia was maintained with nitrous oxide and halothane. An indwelling T-tube was placed in the gallbladder and the common bile duct was ligated; the right femoral vein was cannulated with polythene tubing and a Foley ca-

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theter (no. 8) was placed in the urinary bladder. On recovery the animal was placed in a restraining chair and allowed free access to food and water for the duration of the experiment. ADR (35 mg/m²) was administered as a single IV push, and serial samples of bile and urine were collected.

Male Sprague-Dawley rats weighing 300–450 g were anesthetized with chloral hydrate (40 mg/100 g). A polythene cannula (PE 10) was placed in the common bile duct and the distal end ligated. A similar catheter was placed in the internal jugular vein to facilitate drug administration. The animals were allowed to recover and were observed for 24 h. ADR (4 mg/kg; 25 mg/m²) was then administered via the internal jugular vein and serial samples of bile were collected.

Identification of Parent Drug and Metabolites

Bile. Aliquots of bile (0.2 ml) were extracted with 3 × 2-ml portions of ethyl acetate-1-propanol, 9 : 1 by volume. The combined extracts were evaporated to dryness under a nitrogen jet and were reconstituted with methanol (100 µl) immediately before assay. The extraction efficiency was 90% for ADR and 85% for AMNOL. Portions of the extract (10 µl) were initially examined by TLC with silica gel H plates (5 × 20 cm, 250 µ layer; Analtech, Inc., Newark, Delaware) developed in darkness in chloroform-methanol-water, 80 : 30 : 3 by volume, dried in air, and examined under 254 nm light. Samples were run on the same TLC plate against authentic standards added to control bile and extracted in a like manner. The developing system used allowed adequate separation of polar conjugates and aglycones, as well as ADR and AMNOL. The R_f values were for polar conjugates 0.06, for AMNOL 0.13, for ADR 0.24, and for aglycones 0.94.

Conditions for the HPLC separation and quantitation of ADR and metabolites in bile have already been described [14, 15]. In brief, 5–10 µl aliquots of methanol-reconstituted extracts were analyzed with the aid of a Waters Associates Model ALC/202 liquid chromatograph equipped with a prepacked Whatman Partisil PAC-10 modified normal phase column, a developing system of chloroform-methanol-acetic acid-water, 85 : 15 : 5 : 1.5 by volume, at a flow rate of 2.0 ml/min, and a Schoeffel Instrument Company Model SF-970 flow fluorescence detector with an excitation wavelength of 482 nm and an emission filter (Schoeffel no. 2–73) with low wavelength cutoff near 550 nm. Quantitation of ADR and AMNOL was performed both by weighing peak areas of duplicate samples and by reference to calibration curves constructed from known standards added to bile and extracted as described; for both compounds there was a linear relationship between peak area and concentration. Fresh calibration curves were constructed on each day that assays were performed to compensate for variability in column performance. The coefficient of variation of a standard concentration during the day of assay was 3.2% ($n = 4$). The fluorescence quantum efficiencies of ADR and AMNOL are identical. For the purpose of this study it was assumed that the quantum efficiencies of fluorescent aglycones were similar to those of ADR and AMNOL. The lower limit of sensitivity for ADR and AMNOL with this system is 2–5 pmol.

Urine. Urine samples were analyzed initially by TLC by spotting 25–50 µl of urine directly on silica gel H plates, with development in the solvent system described. Samples of urine (5 µl) were then analyzed directly by HPLC, and quantitation of ADR and AMNOL was performed by weighing peak areas and by reference to calibration curves, as for bile. In addition, the human urine samples were analyzed again following extraction of 2-ml aliquots, as for bile. This last procedure was performed because of the dilute nature of some urine samples. In all instances estimations were carried out in near darkness and in duplicate.

Estimation of Total Fluorescence in Bile and Urine

Total fluorescence was determined by the addition of the same sample volume used for the HPLC assay (5–10 µl) to 1.5 ml of methanol, with fluorescence measured directly in an Hitachi Perkin-Elmer Model MPF-4 corrected spectrum spectrofluorometer (emission wavelength 585 nm on excitation at 485 nm). Concentrations were determined by reference to calibration curves constructed from authentic ADR standard added to both bile and urine and extracted as described.

Results

The cumulative biliary excretion of anthracyclines for man, monkey, and rat during the first 24 h after ADR administration is illustrated in Figs. 1–3. Rats excreted 20% ± 0.27% and 28% ± 1.6% of the administered dose at 24 and 48 h, respectively. With HPLC analysis, the major fraction was ADR. The difference in concentration between total fluorescence (expressed as ADR equivalents) and ADR, as determined by HPLC, was attributed to the presence of AMNOL and other metabolites; with TLC AMNOL and conjugates were seen, but HPLC analysis allowed visualization only of AMNOL. No free aglycones were seen in rat bile.

The cumulative biliary excretion in the patient with complete obstruction was 24% and 30% of the administered dose at 24 and 48 h, respectively. HPLC analysis revealed the presence of ADR, AMNOL, and an additional peak suggesting polar conjugates. The monkey excreted 25% of the administered dose by 24 h and only two peaks, representing ADR and AMNOL, were visualized. For monkey the sum of the concentrations of ADR and AMNOL, as determined by HPLC, at all time points was the same as, or in excess of, the concen-

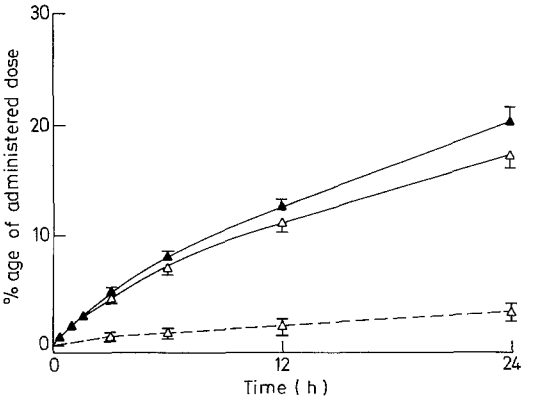

Fig. 1. Cumulative biliary anthracycline exretion in rat (mean ± SE, $n = 4$) following a single IV dose of adriamycin (4 mg/kg or 25 mg/m²). Adriamycin concentrations were determined by HPLC analysis. The curve for adriamycinol and conjugates represents the difference between actual adriamycin levels and total adriamycin equivalents, as determined by total fluorescence. ▲——▲, total fluorescence; △——△, adriamycin; △ - - - △, adriamycinol and conjugates

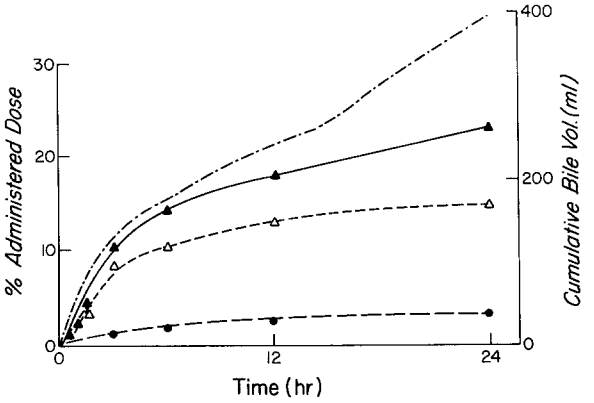

Fig. 2. Cumulative anthracycline biliary excretion in a patient with complete biliary obstruction following a single IV dose of adriamycin (60 mg/m²). Adriamycin and adriamycinol concentrations were determined by HPLC analysis. ▲—▲, total fluorescence; △—△, adriamycin; ●---●, adriamycinol; —·—, cummulative bile volume

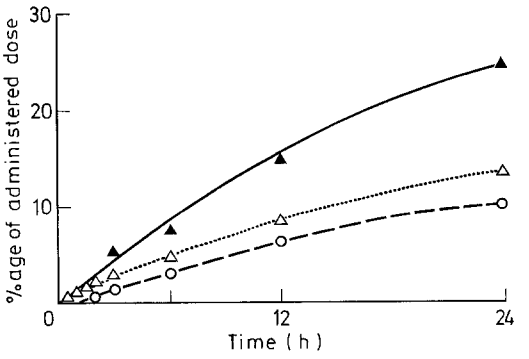

Fig. 3. Cumulative anthracycline biliary excretion in monkey (*Macaca fasicularis*) following a single IV dose of adriamycin (35 mg/m²). Adriamycin and adriamycinol quantities were determined by HPLC analysis. ▲—▲, total fluorescence; △---△, adriamycin; ●—●, adriamycinol

Table 1. Biliary concentrations (nmol/ml) of adriamycin (ADR) and metabolites following a single IV administration of adriamycin to man (60 mg/m²), monkey (35 mg/m²), and rat (4 mg/kg). Total ADR equivalents were determined from total fluorescence and ADR and adriamycinol (AMNOL) by HPLC, as described under Methods

Time[a]	Man[b]			Monkey			Rat[c]		
	Total ADR equivalents	ADR	AMNOL	Total ADR equivalents	ADR	AMNOL	Total ADR equivalents	ADR	AMNOL
0–0.5	11.1	9.0	3.2	28.6	24.1	4.6	56.9	56.9	–
0.5–1	90	95.8	3.4	39.3	25.9	11.2	82.7	83.8	–
1–1.5	205	138	4.9	81.2	55.3	31.5	75.3	72.2	3.1
1.5–3	192	119	8.1	105.8	66.0	48.8	52.2	57.5	4.7
3–6	150	90.1	10.2	98.3	52.4	56.5	34.8	31.1	3.7
6–12	77	48.5	18	71.4	46.7	36.1	26.9	23.5	3.4
12–24	49	25.2	10	64.3	37.2	36.1	20.4	17.0	3.4
24–48	24.5	15.2	8.85	–	–	–	14.4	11.8	2.6

[a] Hours after drug administration
[b] Values are means from two patients
[c] Values are means from four animals

Table 2. Cumulative urinary excretion, expressed as percentages of administered adriamycin (ADR) dose, of ADR and metabolites following a single IV administration of ADR to man (60 mg/m²) and monkey (35 mg/m²). Total ADR equivalents were determined from total fluorescence, and ADR and adriamycinol (AMNOL) by HPLC, as described under Methods

Time[a]	Man[b]				Monkey			
	Urine output (ml)	Total ADR equivalents	ADR	AMNOL	Urine output (ml)	Total ADR equivalents	ADR	AMNOL
0–6	289	3.1	3.1	0.4	4.0	8.7	7.1	0.6
6–12	215	3.7	3.7	0.6	41.5	11.5	11.5	2.3
12–24	455	4.9	4.6	0.8	21.2	12.0	12.8	2.8
24–48	1,096	5.8	6.3	1.2	–	–	–	–

[a] Hours after drug administration
[b] Values are means from two patients

tration determined as ADR equivalents by total fluorescence (Table 1). The same was true for man after 24 h; earlier samples showed the presence of polar conjugates by TLC.

Table 1 shows the biliary concentration of ADR equivalents (total fluorescence) and of ADR and AMNOL (by HPLC) for the three species. The peak concentration of anthracyclines was 0.5–1.0 h in rat, 1.0–1.5 h in man, and 1.5–3.0 h in the monkey. The proportion of ADR metabolized to AMNOL was least in the rat and greatest in the monkey.

The cumulative values for urinary anthracycline excretion in man and monkey are given in Table 2. In both species the sum of the concentration of ADR and AMNOL was the same as, or in excess of, the concentration of ADR equivalents as determined from total fluorescence, except for the 6-h time point in the monkey. HPLC analysis of this fraction indicated that the major component was ADR, with some AMNOL and a polar metabolite, presumably a conjugate. No free aglycones were detected in human or monkey urine.

Discussion

The principal metabolite of ADR is AMNOL, formed by the action of intracellular cytoplasmic aldo-keto reductase [2]. This enzyme is widely distributed throughout the tissues, the highest concentration being found in the kidney [3]. The present study demonstrates that the rate of reduction of ADR by aldo-keto reductase is species-dependent, the lowest activity being in the rodent and the highest in the monkey (Tables 1 and 2). Other species with reportedly high rates of reduction are rabbit and hamster [3, 4]. Since only minimal amounts of AMNOL were detected in the rat, the inference is that in this species ADR is a poor substrate for the enzyme. In the monkey the enzyme is more active, and it is of interest that, in bile, total fluorescence was fully accounted for by ADR and AMNOL. This observation leads us to conclude that in the monkey there are no other fluorescent metabolites of consequence formed for the first 24 h following IV ADR administration. Man appears to be intermediate between rodent and monkey: AMNOL is produced and polar conjugates are formed.

To date there has been only one study on the identification of ADR and metabolites in human bile in a patient who, like our two human subjects, had disseminated malignancy and an indwelling T-tube [18]. Approximately 40% of the administered dose was recovered as fluorescent products in about 300 ml bile at 24 h; ADR accounted for 50%, AMNOL 20%, and the remainder (30%) consisted of a variety of metabolites, including several aglycones. In the present study, 24% of the total dose was recovered as fluorescent products in 406 ml bile in the patient with complete obstruction; ADR accounted for 64%, AMNOL 20%, and the rest (16%) was made up of polar metabolites.

Comparison of two patients by different analytical approaches is difficult, but we feel certain comparisons are valid. Complete obstruction was assumed on radiologic evidence in the patient reported here, but it is possible that collection was incomplete. Comparison of cumulative excretion is therefore unreliable, but comparison of the *proportion eliminated* as fluorescent products is justifiable. The proportions eliminated as ADR and AMNOL were similar, but of the remaining fraction only polar conjugates were identified during the first 24 h. The findings were similar in the patient with incomplete obstruction. The difference may reflect within-species variation in metabolism or, alternatively, differences in methodology. The absence of aglycones in rat, monkey, and human bile suggests that some of the previously reported aglycone metabolites may have resulted from spontaneous degradation, or from the hydrolytic action of silica gel [14].

In monkey urine, the major fraction of anthracycline fluorescence was attributable to ADR and AMNOL. For man, total urinary anthracycline fluorescence could be accounted for as ADR and AMNOL, a result that differs from that of other workers who have reported a variety of metabolites in human urine [21]. These differing observations may again reflect differences in the methodology of separation and estimation of inherently unstable materials, or possibly the existence of an enterohepatic circulation for ADR and metabolites.

The evidence for anthracycline enterohepatic circulation is controversial. An absence of anthracycline fluorescence in plasma and bile following oral administration of ADR to mice and rabbits was noted by Arena et al. [1]. Rats with unaltered hepatobiliary drainage treated with IV daunorubicin showed daunorubicinol, polar metabolites, and several aglycones among ileojejunal contents, but only free aglycones were detected distal to the colon [7]. A plasma metabolite is described that has the characteristics of a metabolite undergoing enterohepatic circulation [11]. On the other hand, in isolated rabbit small intestine with an intact blood supply, absorption of ADR was minimal [10]; polar conjugates might, however, be more readily reabsorbable. In view of our observation of the absence of both free aglycones and polar conjugates in human urine, we do not support an hypothesis of an enterohepatic circulation of anthracyclines. Indeed, the absence of an enterohepatic circulation may also be inferred, in part, from the patient with incomplete biliary obstruction, whose urine contained no free aglycones or conjugates, although conjugates were present in the bile.

This report describes the direct quantitation of ADR and metabolites in bile and urine by HPLC. A previous study used a gradient HPLC system for separation but

quantitated the products by radioimmunoassay [16], the overall procedure being time-consuming and requiring facilities for both HPLC and RIA. It was possible here to quantitate ADR and AMNOL reliably over a wide range of concentrations. Compared with TLC/fluorescence, the HPLC assay was more sensitive and reliable at low concentrations, as it was not necessary to reference simultaneously against a blank sample of either bile or urine, both of which normally contain fluorescent materials that excite at 482 nm, the concentration of which will vary from sample to sample.

We do not dispute that in some instances other fluorescent metabolites of ADR may exist in urine, but, if present, they can only amount to a minute proportion of the administered ADR dose, and their significance is speculative; HPLC indicates clearly that AMNOL is the major metabolite.

The simple, rapid, and highly sensitive HPLC analysis described here for the identification and estimation of ADR and AMNOL now provides a method for evaluating other aspects of ADR pharmacology [8] and for determining the influence of other drugs and/or diseases on ADR metabolism. The fact that we could not account for the total ADR dose indicates either that anthracyclines persist for long periods in tissues or that they are degraded to nonfluorescent species that are currently incapable of detection. This is an area that as yet has not been investigated throughly and may have a bearing on both chemotherapeutic response and toxicity.

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