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Determination of Daunorubicin, Doxorubicin and Their Fluorescent Metabolites by High-Pressure Liquid Chromatography: Plasma Levels in DBA_2 Mice

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Summary. *A rapid and nondestructive analytic method has been developed to separate and quantitate daunorubicin, doxorubicin, and their metabolites in biological fluids. This method combines the efficiency of high-pressure liquid chromatography and the sensitivity of fluorescence monitoring. The drug plasma levels achieved after IV administration of either daunorubicin or doxorubicin at 7 mg/kg into DBA_2 mice were studied. The plasma disappearance curves are biphasic with a half-life of 1 min for the first elimination phase. In urine extracts, 13-hydroxy derivatives represent 80% of the fluorescence after injection of daunorubicin and only 4% after administration of doxorubicin.*

Introduction

The pharmacologic studies of daunorubicin (DNR) and doxorubicin (DOX) require an efficient and nondegradative extraction procedure as well as a sensitive quantitation of the drugs and their metabolites in biological samples.

High-pressure liquid chromatography (HPLC) has been used to determine DNR [5] and DOX [3, 7] in plasma. However, the methods are lengthy and the extraction procedure does not permit the recovery of all the drug present in the plasma.

We describe here a simple and rapid extraction procedure for anthracycline drugs from biological samples, which is efficient and nondestructive. The parent drugs are separated from their metabolites by HPLC and quantified by fluorometry.

Materials and Methods

Daunorubicin (DNR; 13057 R.P.), daunorubicinol (DOL; 20798 R.P.), and doxorubicin (DOX) were provided by Rhône-Poulenc, S.A., Paris, France.

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Female DBA_2 mice (20–22 g) were injected in the tail vein with either DNR or DOX at 7.0 mg/kg. After various times, the blood was collected, during sacrifice, from the femoral vein, on EDTA as anticoagulant. The plasma was obtained by centrifugation at 4° C for 10 min at 2300 rpm (rotor 259, Damon/IEC model PR 6000 centrifuge, Needham Hts., Massachusetts, USA). To 0.1 ml plasma or urine, we added 0.1 ml 0.1 M borate buffer pH 9.8 containing the internal standard (DNR at 10 μg/ml when DOX was analyzed, DOX at 10 μg/ml when DNR is analyzed). Drugs were extracted by 1.8 ml of chloroform : methanol (4 : 1 by volume) and an aliquot of the organic phase injected into the Hewlett-Packard model 1084 high-pressure liquid chromatograph (Hewlett-Packard GMBH, Böblingen, FRG). A Gilson model FL 1 A/B flow fluorometer (Gilson, Middleton, USA) was connected to the chromatograph, and the fluorescence (λ_{exc} 480 nm, λ_{em} 560 nm) signal plotted and integrated by the H-P terminal.

The samples were injected into the 20 μl loop valve of the chromatograph, and the Lichrosorb Si-60 column (Hibar, 25 cm × 3 mm, from Merck, Darmstadt, FRG) was eluted by a mixture of chloroform, methanol, glacial acetic acid, and a 0.3 mM $MgCl_2$ water solution (720 : 210 : 40 : 30 by volume).

Results

As shown in Fig. 1, with the conditions used, a mixture of 30 ng/ml of DNR, DOL, and DOX is separated in less than 7 min at a flow rate of 0.6 ml/min. The peaks are sharp and well resolved. The retention times (t_R) that reflect the polarity of the compounds are, respectively, 2.1 min for the DNR or DOX aglycones (DNRone or DOXone), 4.4 min for DNR, 5.6 min for DOL, and 6.2 min for DOX. From the ratio retention time (t_R)/baseline bandwith (bw), an efficiency of 3100 theoretical plates (N) is calculated for our 25 cm high Lichrosorb Si-60 columns using the following equation [6]:

$$N = 16 \cdot (t_R/bw)^2 .$$

We have shown previously that linear relationships are obtained between the concentrations of DNR, DOL, and DOX, and the integrated area of their respective peaks, when an equimolar mixture of the three com-

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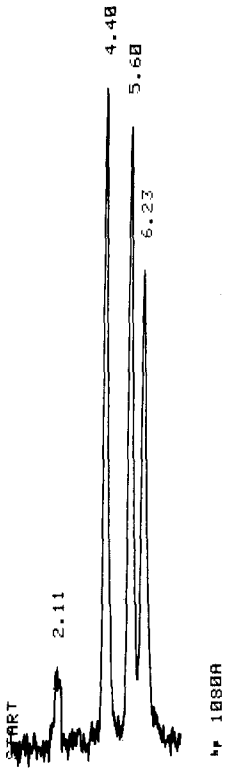

Fig. 1. Separation of daunorubicin, daunorubicinol, and doxorubicin by HPLC. DNR, DOL, and DOX were separated from an equimolar mixture (0.05 nM/ml) by HPLC and estimated by fluorometry as described in Materials and Methods. At a flow rate of 0.6 ml/min, the following t_R were obtained: 4.4 min (DNR), 5.6 min (DOL), and 6.2 min (DOX)

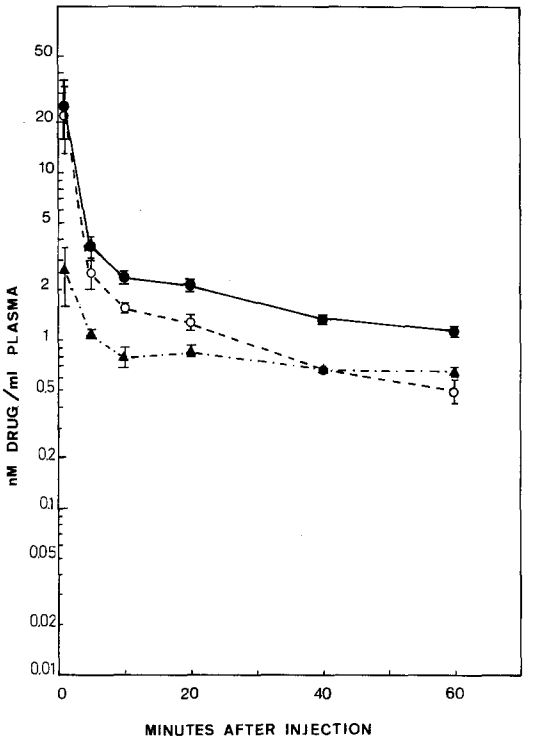

Fig. 2. Plasma levels of daunorubicin and daunorubicinol after IV injection of DNR into DBA_2 mice. DNR was injected IV at 7 mg/kg into DBA_2 mice. After various times, DNR and DOL concentrations in the corresponding individual plasma aliquots were determined as described in Materials and Methods. Mean ± SD of three separate experiments. O---O: DNR; ▲-·-·-▲: DOL; ●—●: total drug fluorescence

Table 1. Recovery of DNR and DOX added to mice blood aliquots[a]

Drug added (μg/ml)	Drug recovered after	
	DNR injection[b,c] (μg/ml)	DOX injection[b] (μg/ml)
5.0	4.9 ± 0.2	5.2 ± 0.3
10.0	9.7 ± 0.6	9.5 ± 0.5
25.0	26.0 ± 0.2	24.5 ± 0.8
50.0	52.9 ± 0.6	50.7 ± 1.9
75.0	73.9 ± 3.9	74.6 ± 3.2
100.0	101.0 ± 3.6	100.3 ± 3.0

[a] DNR or DOX was added to mice blood samples in order to obtain drugs concentrations of 5, 10, 25, 50, 75, and 100 μg/ml
[b] Mean ± SD of four determinations
[c] Sum of DNR and DOL found in the blood (± 16% DOL in each sample)

pounds is injected into the chromatograph in the range of 10—1000 ng/ml [2]. The lowest amount of anthracycline drug detectable is 5×10^{-14} mol corresponding to a concentration of 2.5×10^{-12} mol/ml.

To check our extraction method, either DNR or DOX were added in increasing concentrations to blood samples, in order to obtain drug concentrations of 5, 10, 25, 50, 75, and 100 ng/ml. The drugs were extracted as described in Materials and Methods, and an aliquot of the organic phase injected into the chromatograph. The recoveries are shown in Table 1, and from those results a mean percent recovery of 100.8 ± 3.5 for DNR and 99.7 ± 3.1 for DOX was calculated. The recovery of DNR takes into account the DOL present in blood and which represents about 16% of the total fluorescence of the blood extract. No endogenous fluorescent compounds, which could affect the drug determinations, could be detected in the plasma and urine of DBA_2 mice.

We have used this HPLC method to follow either DNR or DOX and their metabolites in plasma and urine. After DNR injection, the parent drug is the main compound during the first 40 min following the injection (Fig. 2). The first phase of elimination with a half-life of 1.3 min is followed by another phase with a half-life of 24.8 min. After 10 min, the plasma levels of DOL remain at about 0.7 nM/ml, decrease very slowly thereaf-

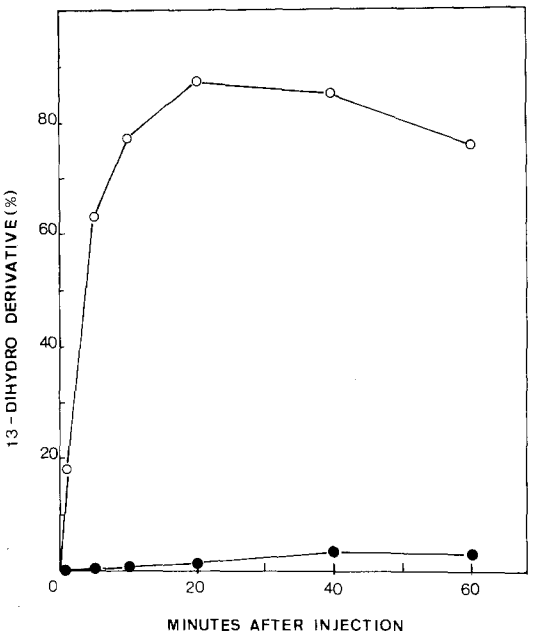

Fig. 3. Daunorubicinol and doxorubicinol in urine samples after IV injection into DBA_2 mice. DNR or DOX was injected IV at 7 mg/kg into DBA_2 mice. After various times, urine aliquots were analyzed as described in Materials and Methods. The results are expressed as a percentage of the total fluorescence: O: DOL found after DNR injection; ●: DOXol found after DOX injection

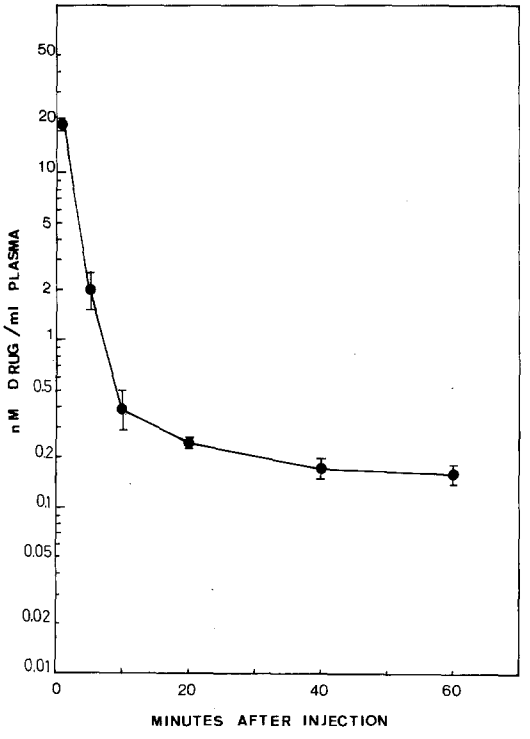

Fig. 4. Plasma levels of doxorubicin after IV injection into DBA_2 mice. DOX was injected IV at 7 mg/kg into DBA_2 mice. DOX concentrations in the corresponding individual plasma aliquots were determined as described in Materials and Methods. Mean ± SD of three separate experiments. ●—●: DOX

ter (half-life: 205 min) and reach higher levels than those of DNR after 40 min. If we add the levels of DNR to those of DOL, the total drug elimination from the plasma is biphasic with a half-life of 1.4 min for the first phase, followed by a slower phase with a half-life of 47 min. In the urine extracts (Fig. 3), DOL is the main compound after 10 min and its percentage levels off at 85% of the total fluorescence after 20 min.

After injection of DOX, the parent drug is the major compound found in the plasma with only a trace amount of doxorubicinol (DOXol) and doxorubicinone (Fig. 4). The plasma levels of DOX decrease rapidly and the first phase of elimination is characterized by a half-life of 1.2 min. The plasma level of DOX, 60 min after the IV injection, is eight times lower than that of DNR. In the urine extracts, DOX is the main compound and DOXol accounts for only 4% of the total fluorescence 60 min after the injection (Fig. 3).

Discussion

The usefulness of the method described to investigate the pharmacokinetics of anthracycline drugs resides in the rapid and efficient extraction procedure, in the good resolution of the peaks, and in the fast HPLC analysis. The extraction procedure permits the recovery of more than 99% of DNR or DOX when they are added to mice blood. The high resolution of the peaks is shown in Fig. 1 by the separation of DNR, 13-OH-DNR (DOL), and 14-OH-DNR (DOX), which is due to the high number of theoretical plates: 3100 for our 25 cm column. The rapidity of the method is illustrated by the fact that it takes at the most 20 min to obtain the chromatogram from the time of blood collection, allowing us to process up to 60 samples daily.

When DBA_2 mice were injected IV with DNR at 7 mg/kg, the plasma decay plots for the drug and its main metabolite (DOL) are at least biphasic. A first elimination phase (parent drug + metabolites) with a half-life of 1.4 min for DNR and 1.2 min for DOX could be determined by taking blood samples at very short time intervals after the injection. The second phase of elimination (parent drug + metabolites), with a half-life of 47 min for DNR and 70 min for DOX, corresponds to the first phase often described for those compounds [8].

Felsted et al. [4] have isolated and purified from rat liver extracts a ketoreductase which reduces the C-13 carbonyl group both of DNR and of DOX. They found that in vitro DNR is a far better substrate than DOX having greater V_{max} and lower K_m values. The percentages of DOL and DOXol we found in the urine extracts suggest that in vivo, too, DNR is a better substrate for the ketoreductase. However, the activity of the enzyme varies from animal to animal and even from strain to

strain, since we found greater percentages of DOL in urine extracts of DBA_2 mice than in urine extracts of NMRI mice after an IV injection of DNR at a similar dosage [1].

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