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Distribution and Metabolism of Rubidazone and Daunorubicin in Mice

A Comparative Study

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Summary. *A comparative investigation of the distribution and metabolism of rubidazone and daunorubicin was conducted in NMRI mice, after a single IV dose of either rubidazone (8.4 mg/kg) or daunorubicin (7 mg/kg). The anthracyclines were analyzed by high-pressure liquid chromatography and fluorometry. Daunorubicin was the main compound found after daunorubicin administration, except in kidney and urine, where daunorubicinol was the main metabolite with trace amounts of aglycone. In contrast, rubidazone undergoes extensive metabolism into daunorubicin, daunorubicinol, and aglycone. The total drug level is significantly lower after rubidazone administration in kidney, heart, gastrointestinal tract, spleen, and urine, and the biliary excretion of rubidazone is more important than that of daunorubicin. When compared with daunorubicin, significantly less daunorubicinol is produced after rubidazone administration.*

Introduction

Daunorubicin (DNR) and doxorubicin (DOX) are used extensively in the treatment of a variety of acute leukemia and solid tumors [5, 6, 13]. Their utility is restricted, however, by the occurrence of severe cardiomyopathy and by bone marrow toxicity.

Among the new anthracycline derivatives, rubidazone (RBZ), the benzoyl hydrazone of DNR [8, 12], retains a therapeutic activity and has a lower toxicity than DOX and DNR [1, 14].

We report here an analytic method, based on high-pressure liquid chromatography and fluorometry to determine RBZ and its metabolites in biological samples. Using this method we have determined the stability of RBZ and analyzed the tissular distribution and metabolism of this drug in several mice tissues.

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Materials and Methods

Rubidazone (RBZ; 22050 R.P.), daunorubicin (DNR; 13057 R.P.), and doxorubicin (DOX) were supplied as hydrochlorides by Rhône-Poulenc, S.A., Paris, France.

Female NMRI mice (average weight, 24 g) were injected in the tail veins with either RBZ at 8.4 mg/kg or DNR at 7 mg/kg. Drugs were dissolved in 0.7 M glycine pH 7.6 buffer.

After various times, the blood was collected, during sacrifice, from the femoral vein, on EDTA as anticoagulant, and plasma obtained by centrifugation at 4° C for 10 min at 2300 rpm (Damon/IEC International Centrifuge, model PR 6000, rotor 259, Needham Hts., Massachusetts, USA). The drugs were detected in the plasma by high-pressure liquid chromatography (HPLC) and fluorometry after extraction of the drugs by chloroform : methanol (vide infra).

Determination of the tissue distribution was performed 30 and 120 min after IV administration of either RBZ (8.4 mg/kg) or DNR (7 mg/kg). Several tissues, blood, bile, and urine were taken and immediately kept in 1.0 ml ice-cold phosphate buffered saline (PBS), pH 7.4. The tissue aliquots were homogenized in 7-ml glass Potter-Elvehjem homogenizers. After rinsing the homogenizer with 1 ml PBS, the pooled suspensions were sonicated for 30 s at 50 W (Sonicator B-12, Branson Sonic Power Co., Danbury, Connecticut, USA). Blood was collected on EDTA as anticoagulant and sonicated in the same manner. The tissular and blood proteins were measured by the Lowry method [9]. The amount of blood contaminating each tissue sample was carefully measured using the immunologic method of Mancini [10] by estimating the concentration of serum albumin present in the tissues. The rabbit antiserum directed against mouse serum albumin was kindly supplied by J. P. Vaerman, Laboratoire de Médecine Expérimentale, I.C.P., Brussels, Belgium. The drug and protein values of each sample were corrected for the amount of contaminating blood. Experiments with perfused livers, using the same immunologic method, indicated that the amount of albumin present in the hepatocytes is negligible as compared with the one due to contaminating blood. The volume of bile, collected from the gall bladder, was estimated by weighing and assuming a specific density of 1.

Drugs were analyzed on 0.1 ml homogenate samples to which 0.1 ml of an internal standard (DOX at 10 μg/ml borate pH 9.8 buffer) was added. The drugs were extracted by addition of 1.8 ml of chloroform : methanol (4 : 1 by volume). The tubes were shaken and an aliquot of the organic layer injected into the chromatograph. A Gilson Fl 1 A/B fluorometer (Gilson, Middleton, USA) was connected to a Hewlett-Packard model 1084 high-pressure liquid chro-

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matograph (Hewlett-Packard, GMBH, Böblingen, FRG), using interference filters of 520 nm and 600 nm for excitation and emission wavelengths, respectively. The prefilled column (250 mm × 3 mm, Brownlee Labs, Italy) contained 10 μ Lichrosorb DIOL modified silica gel particles and was eluted with a mixture of chloroform, methanol, pH 7.5 glycine buffer, 0.3 mM in $MgCl_2$ (720 : 280 : 30) at a flow rate of 1.0 ml/min.

Results

We have adapted a method previously described [2] to separate RBZ from its metabolites and degradation products, and quantify them by fluorometry. With an eluent mixture containing glycine buffer instead of acetic acid, at a flow rate of 1.0 ml/min, the retention times were 2.8 min for daunomycinone (DNRone), 5.6 min for RBZ, 6.1 min for DNR, and 8.0 min for DOX used as internal standard (Fig. 1). A retention time of 7.2 min was observed for daunorubicinol (DOL) using the same conditions.

The stability studies of RBZ are illustrated in Fig. 2. The RBZ powder when dissolved in chloroform : methanol (4 : 1 by volume) and injected directly into the chromatograph was found to contain only 1–2% DNR. When dissolved in citrate buffer at pH 4.5, RBZ hydrolyzes rapidly in DNR (Fig. 2A). The hydrolysis follows a first-order kinetic with a half-life of 15 min (insert, Fig. 2A).

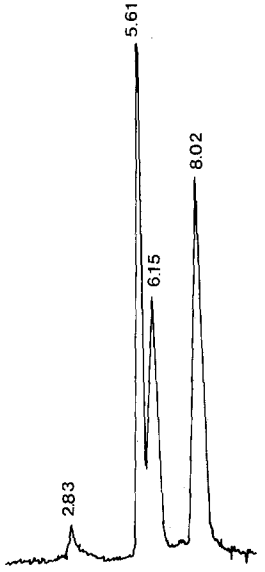

Fig. 1. Separation of daunomycinone, rubidazone, daunorubicin, and doxorubicin by high-pressure liquid chromatography. DNRone, RBZ, DNR, and DOX were separated from a mixture by HPLC and estimated by fluorometry as described in Materials and Methods. With a flow rate of 1.0 ml/min, the following retention times were obtained: 2.83 min (aglycones), 5.61 min (RBZ), 6.15 min (DNR), and 8.02 min (DOX)

The stability of RBZ depends on its concentration and on the strength of the buffer used. At a high concentration of 25 mg/ml 0.7 M glycine pH 7.6 buffer, the pH of the solution was found to be 6.1, and at this pH RBZ hydrolyzes rapidly into DNR. RBZ is more stable at a lower concentration of 20 μg/ml 0.1 M glycine pH 7.6 buffer. As shown in Fig. 2B, 50% hydrolysis is reached after 33 h in 0.1 M glycine buffer (pH 7.6), while it takes 50 h in calf serum (pH 7.7), 97 h in bidistilled water (pH 6.6), and 150 h in 0.9% NaCl (pH 6.7).

When RBZ is injected IV into NMRI mice at 8.4 mg/kg, no DNR or DOL is found in the plasma even after 40 min. The disappearance of RBZ is at least biphasic (Fig. 3A). The first phase of elimination has a half-life of 2.2 min, which is greater than the half-life (0.4 min) of the corresponding phase for DNR (Fig. 3B).

In the urine, an appreciable amount of drug appears only 5 min after the IV injection, and since the urinary flow is unknown, relative metabolites concentrations are expressed in percent of total fluorescence. After RBZ administration (Fig. 4A), RBZ percentage decreases

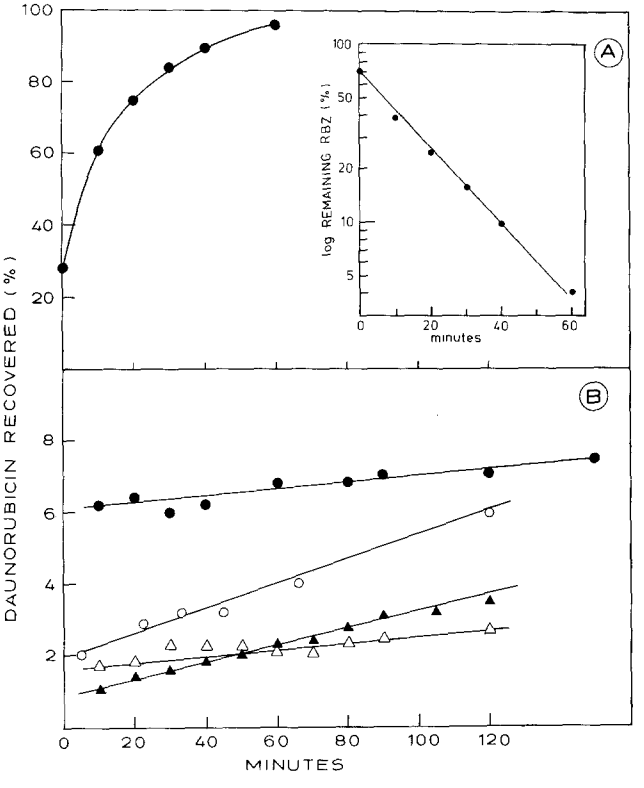

Fig. 2. Stability of rubidazone in aqueous solutions. Rubidazone solutions at 20 μg/ml were incubated at 22° C at acid or neutral pH. After various times, the amount of DNR present in the solutions was determined by HPLC as described in Materials and Methods. (**A**) Stability in citrate buffer 0.2 M, pH 4.5 (●); *insert:* percent of remaining RBZ in solution (●). (**B**) Stability in bidistilled water (●), glycine buffer 0.1 M, pH 7.6 (○), calf serum (▲), and NaCl 0.9% (△)

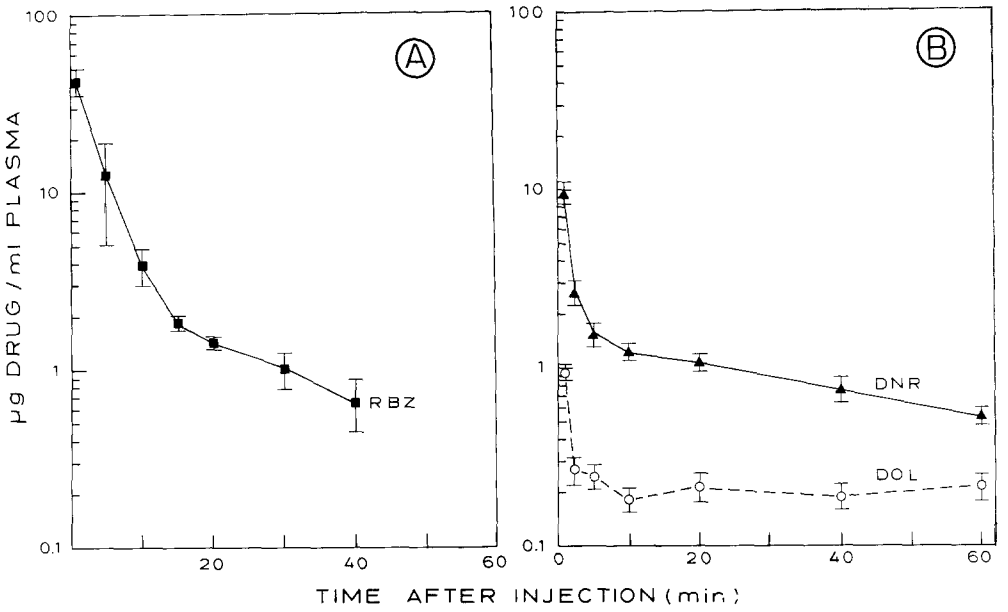

Fig. 3. Plasma levels of rubidazone and daunorubicin after IV injection into NMRI mice. RBZ and DNR were injected IV and, after various times, drug concentrations in the corresponding individual plasma samples were determined as described in Materials and Methods. Mean ± SD of three separate assays are given. **(A)** RBZ injected at 8.4 mg/kg; RBZ (■); **(B)** DNR injected at 7.0 mg/kg; DNR (▲—▲) and DOL (O - - O)

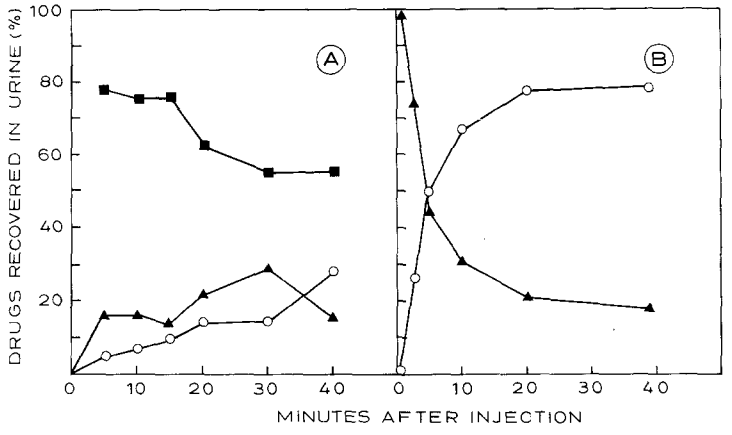

Fig. 4. Metabolites found in urine extracts of NMRI mice injected IV with either rubidazone or daunorubicin. RBZ and DNR were injected IV and drug concentrations in the corresponding urine extracts were determined as described in Materials and Methods. The results are expressed in percentage of the total fluorescence. **(A)** RBZ injected at 8.4 mg/kg; RBZ (■), DNR (▲), and DOL (O); **(B)** DNR injected at 7.0 mg/kg; DNR (▲) and DOL (O)

from 80 to 60% in 40 min, DNR percentage remains constant between 15 and 25%, and DOL percentage increases up to 30% after 40 min. After DNR administration, however (Fig. 4B), the DOL percentage reaches 90% after 60 min.

The levels of drug and metabolites in various tissues and biological fluids, 30 and 120 min after IV administration of either RBZ or DNR, are illustrated in Fig. 5 and 6.

Tissues accumulating RBZ and its metabolites after 30 min, in order of decreasing concentrations, are: liver, duodenum, lung, kidney, heart, colon, stomach, spleen, and brain (Fig. 5). After 120 min, the amount of RBZ in the organs generally decreases, except for the spleen. By comparison with DNR administration, the drug levels in kidney, heart, colon, stomach, and spleen are lower after RBZ administration and significantly greater in liver (Fig. 6).

No significant differences in whole blood levels are seen either 30 min or 120 min after IV injection of RBZ or DNR. Differences were found, however, in urinary and biliary excretion. The biliary excretion of RBZ and metabolites is five times more important than for DNR. In urine, the levels of RBZ and metabolites are three times lower than those of DNR (Fig. 6).

Unmetabolized RBZ is the main compound in blood and liver, while in the other organs RBZ is rapidly transformed into DNR and DOL.

Aglycones are important metabolites in the gastrointestinal tract, whole blood, and in brain. The DOL level, 30 min after the IV injection of RBZ, is generally low, except in kidney and urine. After 120 min the parent

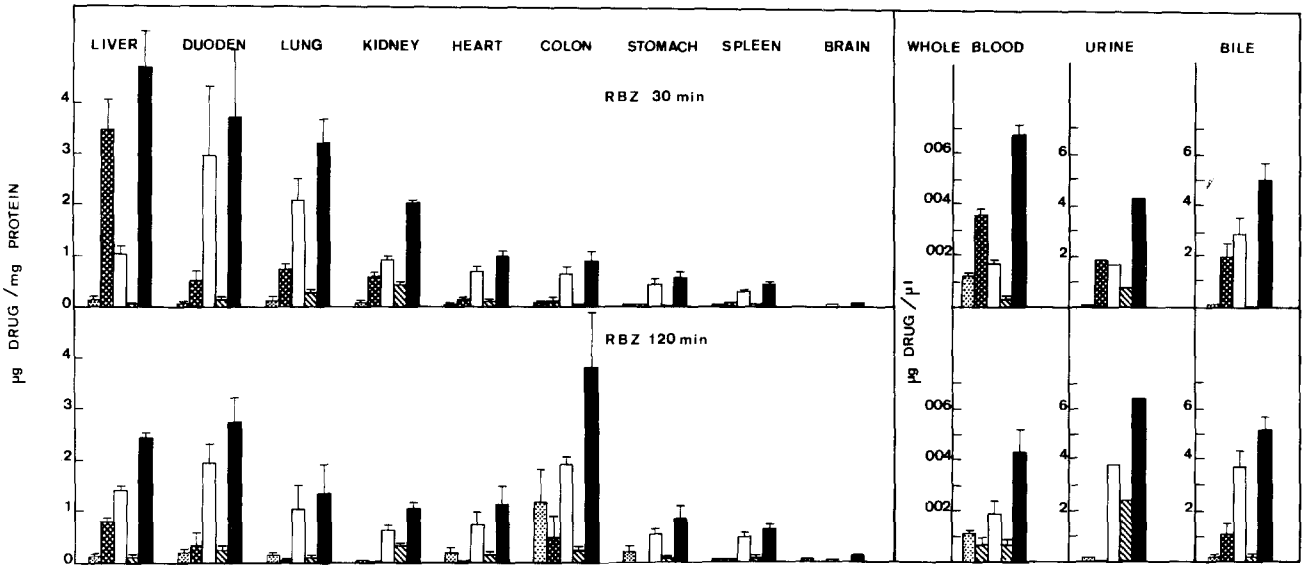

Fig. 5. Tissue distribution of aglycones, rubidazone, daunorubicin, and daunorubicinol 30 and 120 min after IV administration of rubidazone into NMRI mice. Drug concentrations were determined in several tissues 30 and 120 min after IV injection of RBZ at 8.4 mg/kg. Mean ± SE of four different experiments are given. ▨: aglycones; ▩: RBZ; □: DNR; ▧: DOL; ■: sum of parent drug and metabolites

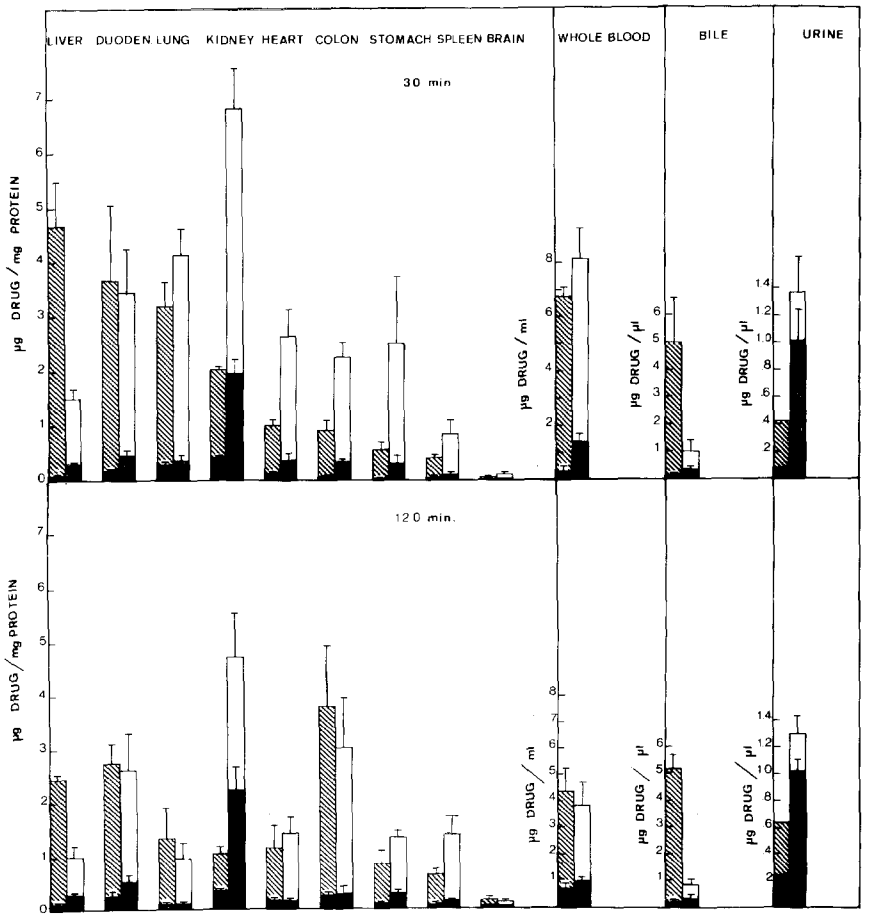

Fig. 6. Tissue distribution of rubidazone, daunorubicin, and daunorubicinol, 30 and 120 min after IV administration of either rubidazone or daunorubicin into NMRI mice. Daunorubicinol and total drug concentrations were determined in several tissues 30 and 120 min after IV injection of RBZ at 8.4 mg/kg or DNR at 7.0 mg/kg. Mean ± SE of four different experiments in the case of RBZ; mean ± SE of five different experiments in the case of DNR. ▧: parent drug + metabolites concentrations after RBZ injection; □: parent drug + metabolites concentrations after DNR injection; ■: DOL concentrations either after RBZ or DNR injection

drug is still found in whole blood, urine, liver, and gastrointestinal tract (Fig. 5).

We have found that in comparison with DNR, significantly less DOL is present in the tissues after IV administration of RBZ. The percentage of DOL after RBZ administration is about 20 times lower in bile and liver, and about four times lower in whole blood, urine, and gastrointestinal tract. After 120 min the percentage of DOL remains significantly lower in liver, bile, and urine. By comparison with DNR also, the percentages of aglycones are about ten times lower in bile, kidney, and duodenum, and about three times lower in liver, colon, and stomach.

Discussion

RBZ has been found to be relatively stable only in dilute solutions and at neutral pH. At higher concentrations and in inadequate buffer conditions, RBZ is unstable and generates DNR. The hydrolysis of RBZ observed in vitro at acidic pH occurs also in vivo and could at least partially be due to a transit of RBZ through the intracellular acidic compartments, like the lysosomes.

The tissue distributions of RBZ and DNR are quite different. Drug levels (RBZ, DNR, or DOL) in some tissues (kidney, heart, colon, stomach, and spleen) are lower 30 min after administration of RBZ probably as a result of a greater biliary excretion of RBZ. The lower amounts of RBZ obtained in some tissues after RBZ injection can explain the lower toxicity of this compound. For instance, the exposure of heart muscle to RBZ is about three times lower than the exposure to DNR. As the cardiotoxicity is probably related to the heart exposure of the drugs, RBZ is expected to be less cardiotoxic than DNR. Such evidence has been obtained experimentally on rats [14], using ECG changes as criteria, and has been confirmed in histopathologic studies on rabbits. (R. Jaenke, personal communication). The lower cardiotoxicity of RBZ has also been confirmed in clinical trials by Benjamin et al. [4]. It remains to be established, however, whether the decrease in toxicity of RBZ is accompanied by an increase in its therapeutic index, since it has been shown that higher doses of RBZ are required to obtain the same therapeutic activity as with DNR [7, 11].

It has been postulated by Benjamin [3] that the lower DOL level found in human plasma after RBZ administration could be due to an inhibition of the ketoreductase by RBZ. We found in mice less DOL in plasma, blood, urine, and various tissues after IV administration of RBZ, but this could be explained by the lower amount of DNR able to be reduced by the enzyme, since in order to be reduced, RBZ must first be hydrolyzed into DNR.

After injection into NMRI mice of an 'aged' RBZ solution, containing 75% DNR, 50% of the DOL was present after 1 h in the urine extract. This percentage could be explained entirely by the amount of DNR present in the injected solution.

RBZ can thus be viewed as an acid-labile prodrug of DNR which, because of a different tissue distribution and a different metabolism, becomes less toxic when compared with DNR.

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