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Pharmacokinetics and tissue distribution of doxorubicin-loaded polymethacrylic nanoparticles in rabbits

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Summary

The anticancer drug, doxorubicin (DXR), was coupled to polymethacrylic nanoparticles having an average diameter of 0.3 μm. Using new methods for the determination of doxorubicin and its main metabolites in plasma and tissue samples, blood clearance and organ deposition of doxorubicin-loaded nanoparticles were studied in the rabbit after intravenous injection. It was shown that adsorption of DXR on the nanospheres caused a prolonged elevation in DXR plasma levels and a reduction of the total clearances. However, no statistical differences were observed between the pharmacokinetic parameters determined after intravenous administration of doxorubicin linked to nanoparticles, coated or not with a non-ionic surface-active agent. One hour after the injection of doxorubicin-bound nanospheres, the concentration of DXR in the liver was found to be 75% higher and the levels of both metabolites were 2-fold lower than those corresponding to the free injected drug. Modifications of the concentrations of DXR and its metabolites were also demonstrated in the kidneys after administration of DXR-loaded nanoparticles. As pharmacokinetics, organ distribution and metabolism of nanoparticle-bound doxorubicin became modified, the activity of polymethacrylic nanospheres as intrahepatic slow-release systems and their potential therapeutic application as new drug-carriers for anthracycline targeting are discussed.

Introduction

The anthracycline antibiotic, adriamycin (doxorubicin), is one of the most important cytostatic drugs used in the field of cancer chemotherapy (Blum and Carter, 1974; Di Marco, 1975; Bender et al., 1978; Young et al., 1981). Studies have shown that the aminoglycosidic anthracycline intercalates into the DNA strand and inhibits further DNA and RNA biosynthesis (Di Marco, 1975; Goodman et al., 1977; Schwartz, 1975). In addition, other mechanisms of action are also ascribed to doxorubicin: membrane binding and lipid peroxidation (Murphree et al., 1976; Myers et al., 1977; Tritton et al., 1978; Tritton and Yee, 1982; Tokes et al., 1982).

Doxorubicin is usually used in the treatment of neoplastic diseases, such as leukemias and various solid tumors, but it induces acute hematologic and dose-dependent cardiac toxicity (Benjamin, 1975; Lenaz and Page, 1976; Billingham et al., 1978;

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Reich, 1978; Van Vleet et al., 1980). So, we developed a new injectable drug delivery system, consisting of polymethacrylic nanoparticles (Rolland et al., 1986a, 1987; Rolland, 1987) allowing a passive targeting of drugs bound to these nanospheres, with the aim of reducing the toxic effects whilst increasing the therapeutic activity of doxorubicin.

The present report describes the pharmacokinetic modifications observed after intravenous administration of doxorubicin-loaded nanoparticles in the rabbit, referring to free injected doxorubicin. Then, in order to explain the previous changes, adriamycin and metabolite concentrations are evaluated in different organs.

Materials and Methods

Preparation of polymethacrylic nanoparticles

The nanoparticles were prepared as previously described (Rolland et al., 1986a and b; Rolland, 1987) by aqueous emulsion copolymerization of methacrylic monomers; methyl methacrylate (MMA), 2-hydroxypropyl methacrylate (HPM), methacrylic acid (MA) and ethylene glycol dimethacrylate (EGD) (Merck, France). The monomers were purified, before use, by distillation as explained elsewhere (Rolland et al., 1986a). The monomers (MMA, 1.4 ml; HPM, 0.75 ml; MA, 0.25 ml; EGD, 0.10 ml) were introduced, with stirring, into 97.5 ml of an aqueous solution of emulsifier (0.001% of Pluriol PE 6800, BASF, France) or into 97.5 ml of distilled water. 10 mg of potassium persulfate (Merck, France) was added as free radical initiator and the copolymerization was maintained for 1 h at 90 °C. After filtration (glass filter, 10–20 μm), the milky suspension was dialysed to remove the remaining monomers and other ionic impurities (Rolland et al., 1986c). The size of the nanoparticles was assessed using a Coulter Nano-Sizer (Coultronics, France) to 0.3 μm and their morphology and internal structure were examined by scanning and transmission electron microscopy (Rolland et al., 1987). Finally, both suspensions of nanospheres were adjusted to pH 8.4 (0.01 M CH_3COONa; 1 N NaOH) and brought to isotonicity with glucose.

Injectable preparations of doxorubicin

The standard solution of adriamycin (free doxorubicin) was obtained by dissolution of 10 mg of drug (Adriblastine, Laboratories R. Bellon, France) in 10 ml of a 5% aqueous glucose solution. On the other hand, an aqueous solution of doxorubicin (10 mg/ml) was dropped with stirring to the nanoparticles to give a final concentration of 1 mg of drug/ml suspension, with a 96% adsorption yield.

Drug administration to the rabbits

Free and nanoparticle bound doxorubicin (with "B-DXRs" or without surfactant "B-DXR") were injected via the marginal ear vein to New Zealand White male rabbits, weighing 4.4 ($\pm$0.3) kg, the dose corresponding to 1.54 mg/kg and the perfusion speed to 1.16 ml/min. Two groups of 6 rabbits each were used for the pharmacokinetic study, receiving first the solution of free doxorubicin and two weeks later either the "B-DXRs" or the "B-DXR" suspensions at the same dose. For the tissue distribution analysis, only one rabbit was used for each time.

Plasma samples

Heparinized blood samples (1 ml) were collected from the marginal vein of the contralateral ear immediately after the injection (T_0) and at various times (from 5 min to 6 h). They were then centrifuged and the plasma was separated and frozen at -20 °C until analysis was performed.

Tissue distribution

After intravenous administration of free or nanoparticle-bound doxorubicin (without surfactant) the rabbits were killed at various times (1, 6, 24 and 96 h), then some organs were removed (liver, spleen, kidneys, heart, lungs), rinsed in distilled water and frozen at -20 °C until assayed.

Analytical methods

Some analytical approaches have been described for the quantitative determination of anthracyclines in biological systems, such as direct fluorescence or radioimmunoassay (Bachur et al., 1976) for total measurement in plasma, thin layer chromatography (Chan and Harris, 1973; Be-

njamin et al., 1973; Israel et al., 1978a; Chan and Wong, 1979) or high-performance liquid chromatography with different extraction methods (Israel et al., 1978b; Eksborg, 1978; Eksborg et al., 1978; Pierce and Jatlow, 1979; Baurain et al., 1979; Averbuch et al., 1981; Oosterbaan et al., 1984; Van Lancker et al., 1986). The new analytical methods, described in this paper, are original and allow the dosage of doxorubicin in plasma and of parent drug and its fluorescent metabolites in different tissues.

Assay of plasma samples. To 0.5 ml of plasma sample, 0.1 ml of internal standard (daunorubicin aqueous solution at 1 μg/ml) and 0.25 ml of 0.5 M Tris buffer pH 8.6 were added. The sample was then extracted with 4 ml of ethyl acetate (Merck, p.a.), by mixing for 4 min on a horizontal shaker; after centrifugation for 5 min at 4000 rpm, the upper organic phase was collected in mini-vials (Interchim, France). 100 μl of 0.1 N sulfuric acid was mixed with the organic phase for 2 min on a horizontal shaker and after centrifugation for 5 min at 3000 rpm, the upper phase was removed by aspiration; the aqueous lower phase (100 μl) was quickly transferred in a tube in which 100 μl of a 0.2 M methanolic solution of sodium acetate was previously dried. At this stage, the aqueous extract presented a suitable pH for its injection into the reversed-phase high-performance liquid chromatographic system (automatic injector Wisp 710 B, constant-flow pump 6000 A, a 254 nm fixed-wavelength detector model 240 (Millipore-Waters, France) and a recorder Omniscribe (Houston Instruments, U.S.A.). 40 μl aliquots were injected into the chromatographic column (μBondapak C 18 column, Millipore-Waters, France) put in a water-bath at 30 °C. The mobile phase consisted of methanol/0.01 M aqueous solution of sodium acetate/acetic acid (70 : 30 : 1 v/v/v), and the flow rate was 1 ml/min. The absorbance of the eluate was measured at 254 nm and quantitation was based on peak-height ratios measurement (doxorubicin/daunorubicin).

Doxorubicin and metabolite tissue extraction and quantitation. The freeze-dried organs were reduced to powder in a mortar, then 100 mg of the powder was mixed with 2 ml of distilled water for liver, spleen, kidneys and 3 ml for heart and lungs. The homogenates were sonicated with an ultrasonic cell disruptor (Vibra Cell, Sonics and Materials, U.S.A.), for 5 min at 150 W with an alternative mode. 100 μl of sonicated suspension was put into 1.5 ml polyethylene conical centrifuge tubes (Treff, Bioblock Scientific, France) and 20 μl of distilled water was added to the assays and 20 μl of standard solutions of doxorubicin to the calibration samples. 100 μl of a 20% trichloroacetic acid solution in a mixture of acetonitrile/distilled water (40/60 v/v) containing 10 μg/ml of desipramine (Ciba-Geigy Lab., France) was finally mixed with the previous suspension on a vortex mixer for 30 s. After centrifugation, 50 μl of the supernatant was quickly transferred into a tube in which 150 μl of a 0.2 M methanolic solution of sodium acetate was previously dried.

20 μl aliquots were injected into the HPLC system (μBondapak C 18 column at 30 °C, automatic injector Wisp 710 B, constant flow-pump 6000 A (Millipore-Waters, France); fluorescence detector GM 970 (Schoeffel Instruments, U.S.A.) set at excitation wavelength of 247 nm and emission wavelength of 550 nm). The mobile phase consisted of methanol/0.01 M aqueous solution of sodium acetate/acetic acid (55 : 45 : 1 v/v/v), and the flow rate was 1 ml/min. Quantitation was based on peak height measurement and tissue concentrations were determined as doxorubicin equivalents for the metabolites because of the absence of standards.

Results and Discussion

Quantitative determination of doxorubicin in plasma

Using the extraction scheme described above, the recoveries of the drugs in plasma were respectively 87% for doxorubicin and 88% for daunorubicin, these values depending on the pH of the 0.5 M Tris buffer. The lowest detectable amount for adriamycin was 5 ng of anthracycline/ml plasma. The standard curves for doxorubicin with daunorubicin as internal standard were linear from 10 to 1000 ng/ml, with a correlation coefficient always better than 0.9990. The retention times were for doxorubicin 8 min and for daunorubicin 12 min (Fig. 1). This ana-

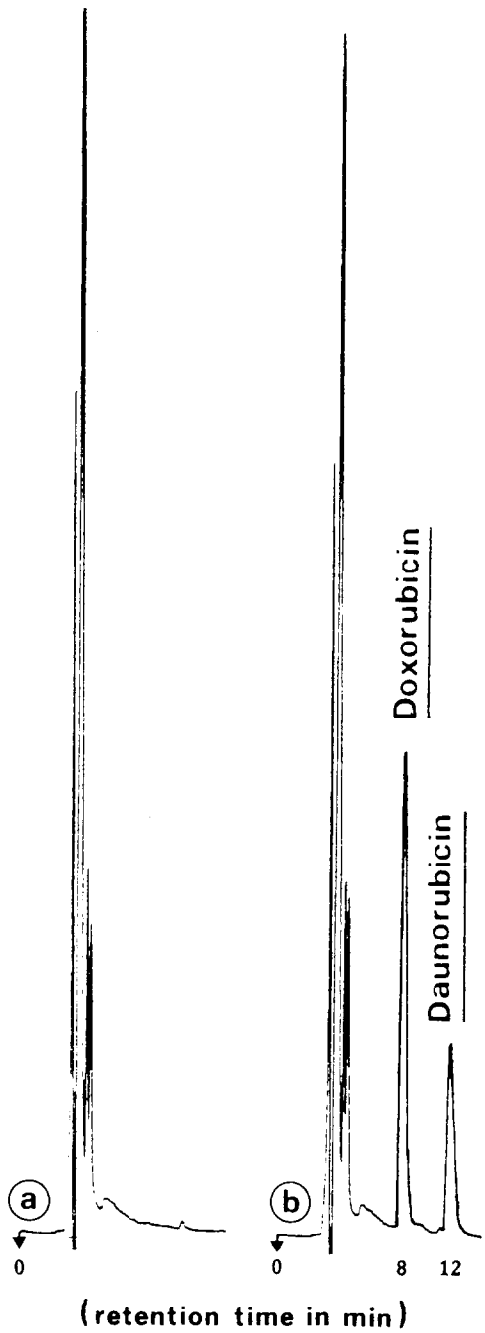

Fig. 1. a: chromatogram of blank plasma. b: chromatogram of a plasma extract: HPLC separation profile of doxorubicin and daunorubicin (internal standard). Extraction and chromatographic conditions are given in Materials and Methods section.

lytical method was reproducible, showing a within-day coefficient of variation of 1.9% for doxorubicin ($n = 10$).

Tissue quantitation of doxorubicin and its two main metabolites

The recoveries of doxorubicin in various organs were measured for two concentrations, 0.4 and 2 μg anthracycline/ml homogenates, and they are shown in Table 1.

The selectivity of the chromatographic technique is illustrated for the kidneys in Fig. 2, and the retention times were: doxorubicinol (13 min), doxorubicin (16.5 min) and doxorubicinone (19.5 min). The standard curves for adriamycin were linear from 0.2 to 2 μg of drug/ml homogenates, with correlation coefficients of 0.9987 (liver), 0.9993 (spleen), 0.9998 (kidneys), 0.9997 (heart) and 0.9983 (lungs). The lowest detectable amounts for doxorubicin were 0.57 μg of drug/g of liver, 0.44 μg/g of spleen, 0.44 μg/g of kidneys, 0.65 μg/g of heart and 0.63 μg/g of lungs. The analysis of 10 liver samples, each containing 2.30 μg adriamycin/g wet weight organ, showed a within-day coefficient of variation of 2.66%.

The analytical methods described above for the determination of doxorubicin and its principal metabolites in biological samples are rapid, reliable, reproducible and highly sensitive and they can be applied to comparative pharmacokinetic and tissue distribution studies.

Pharmacokinetics

Following a 1.54 mg/kg intravenous injection of free or bound adriamycin (B-DXRs and B-DXR) in rabbits ($n = 6$), Fig. 3 shows the plasma concentration–time profiles of the parent drug, corresponding to biphasic curves. They indicated a first short distribution phase (≈ 1.6 min) followed by an elimination phase with a relatively prolonged half-life (≈ 2–3 h). Some pharmacokinetic parameters were valued according to a two-compartment model (Table 2) and statistically compared using the Student's t-test.

Following a 1.54 mg/kg intravenous injection of F-DXR in rabbits, the obtained biphasic curves (Fig. 3) were quite similar to those described by other authors, in rabbits (Bachur et al., 1974) or in

TABLE 1

Recovery of adriamycin (%) in different organs

Doxorubicin concentration (μg/ml homogenates)	Recovery (%)				
	Liver	Spleen	Kidneys	Heart	Lungs
0.4	82.35	79.41	83.82	94.12	91.18
2	93.84	84.59	85.15	95.24	92.20

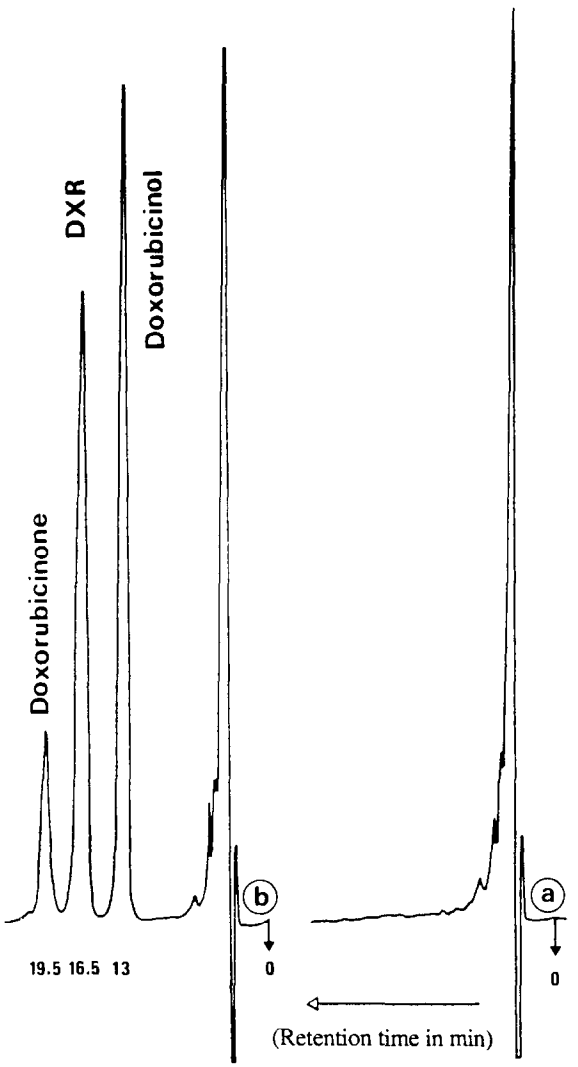

Fig. 2. a: Chromatogram of blank kidney extract. b: HPLC separation of doxorubicin and its metabolites in kidney extract, 1 h after a 1.54 mg/kg intravenous injection of doxorubicin solution in rabbits. Extraction and chromatographic procedures are described in Materials and Methods section.

other animal species (Yesair et al., 1972; Harris and Gross, 1975; Baurain et al., 1979). The disappearance of DXR from the plasma is at least

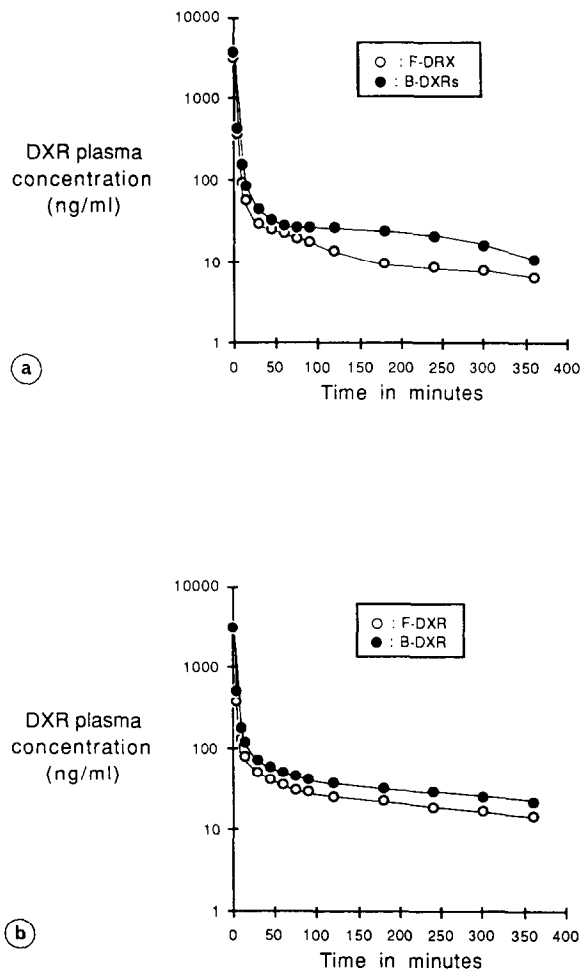

Fig. 3. Mean plasma levels of doxorubicin ($n = 6$) after a 1.54 mg/kg intravenous injection of free and bound DXR (a: B-DXRs group; b: B-DXR group) in rabbits. (The low S.E.M. values are not represented.)

biphasic and was sometimes even described as triphasic (Harris and Gross, 1975; Chan et al., 1978). The rapid distribution phase is characterized by a half-life of about 1.5 min and the total clearance is high, 4.2 and 5.8 l/h/kg, these values being in agreement with those calculated by Bachur et al. (1974), ≈ 4.0 l/h/kg. This total clearance corresponds essentially to a hepatic clearance (Harris and Gross, 1975; Chan et al., 1978) and the primary elimination pathway for DXR is via the hepatobiliary route, while the urinary excretion of this anthracycline has been reported to be about 2% of the dose over an 8-h period in rabbits (Bachur et al., 1974) and 3% after 96 h in mice (Israel et al., 1978a).

For the B-DXRs and B-DXR groups, the plasma levels were always higher than those observed for F-DXR, corresponding to previous results obtained by Couvreur (1983) and by Grislain et al. (1985) for drugs associated with polyalkylcyanoacrylate nanoparticles. Nethertheless, most of the pharmacokinetic parameters were not statistically different after injection of free or bound DXR, except for the areas under the plasma concentration–time curve extrapolated to $t = \infty$ (Table 2), therefore for the total clearances, estimated according to the equation: $CL_{tot} = \text{Dose}/AUC_{\infty}$ (5.84 l/h/kg for F-DXR compared to 3.84 l/h/kg for B-DXRs group; 4.20 l/h/kg for F-DXR and 3.31 l/h/kg for B-DXR group). In another comparative study between entrapped DXR in anionic liposomes and free DXR, both injected in mice, Forssen and Tokes (1983) also observed an increase of the area under the plasma concentration versus time curve with the encapsulation of DXR and a reduction in its volume of distribution. These results can be explained by a modification in the natural distribution of DXR, the carrier leading to the targeting of the drug to cells of the Mononuclear Phagocyte System, predominantly to the liver. Considering the intense specific distribution of the methacrylic copolymer nanoparticles to the liver, the observed pharmacokinetic modifications are less important than those expected; but the plasma drug concentrations only account for the global distribution of the drug, however, without indications about the sites and the intensity of this distribution. So in this case, the qualitative distribution difference of DXR linked to nanoparticles only leads to modified total clearances, and for these new drug delivery systems, consisting of colloidal drug carriers, pharmacokinetic studies are not sufficient to prove important changes in the distribution and/or in the metabolism of associated drugs.

Besides, the pharmacokinetic results obtained for B-DXRs and B-DXR groups are quite similar (Table 2). This comparative study between both nanoparticle suspensions, with or without surfactant, was realized because some authors had demonstrated that the blood clearance and organ deposition of respectively polystyrene and polymethylmethacrylate nanoparticles (Illum and Davis, 1984; Leu et al., 1984) could be modified by coating the particles with a non-ionic surface-active agent. The blood level–time profiles showed that the blood clearance of the surfactant-coated particles was slower than that of uncoated particles. In the present work, no statistical pharmacokinetic differences between B-DXRs and B-DXR groups were observed, probably because of the higher hydrophilicity of the carriers and of the

TABLE 2

Pharmacokinetic parameters of adriamycin after a 1.54 mg/kg intravenous administration of free or bound doxorubicin in rabbits

Parameters	Group I ($\bar{m} \pm$ S.E.M.)				Group II ($\bar{m} \pm$ S.E.M.)			
	F-DXR		B-DXRs		F-DXR		B-DXR	
$T_{1/2\,distribution}$ (min)	1.53	(0.09)	1.66	(0.21)	1.57	(0.08)	1.77	(0.07)
$T_{1/2\,elimination}$ (min)	116.35	(8.40)	154.73	(34.80)	150.62	(10.42)	155.64	(6.41)
Volume of distribution (l/kg)	0.51	(0.10)	0.44	(0.05)	0.53	(0.08)	0.50	(0.03)
AUC_{∞} (μg/l/min)	16467.72	(1661.42)	24753.71	(2906.53)	22846.54	(1929.75)	29782.94	(2021.69)
CL_{tot} (l/h/kg)	5.84	(0.71)	3.87	(0.37)	4.20	(0.45)	3.31	(0.13)

lower rate of non-ionic surfactant (0.001% w/v) compared with the other studies, 1% (w/v) for polystyrene and 0.5% (w/v) for polymethylmethacrylate particles.

Tissue distribution of free and bound doxorubicin

To explain the modified total clearances, observed in the pharmacokinetic studies after injection of doxorubicin bound to the nanospheres in rabbits, the concentrations of doxorubicin (DXR), doxorubicinol (DXol) and doxorubicinone (DXone) were valued in some organs at various times. One hour after a 1.54 mg/kg intravenous injection of free or bound doxorubicin (B-DXR group) in rabbits, corresponding approximately to the end of the distribution phase observed previously, the main differences appeared in the two elimination organs of doxorubicin, the liver and the kidneys (Table 3). The two major metabolites, identified as DXol and DXone, were only detected in liver and kidneys, as already observed by other authors (Bachur et al., 1974; Chan and Wong, 1979), and not in heart, spleen and lungs.

One hour after the administration of doxorubicin bound to the nanospheres, the concentration of DXR in the liver is 75% higher than that obtained for the free injected anthracycline. Inversely, the levels of metabolites (DXol and DXone) are 2-fold lower for the liver one hour after the injection of the bound DXR compared to the free DXR (Table 3). Consequently, the ratios DXR/DXol and DXR/DXone are respectively 4- and 3-fold higher for the liver 1 h after administration of doxorubicin linked to nanospheres compared to the commercialized doxorubicin solution. One hour after intravenous administration of B-DXR, the higher concentrations of DXR and lower levels of DXol and DXone found in the liver compared to F-DXR-injected rabbits (Table 3) are probably due to a hepatic anthracycline targeting, corresponding to the specific and rapid distribution of the polymethacrylic nanoparticles to the liver (60% of injected nanospheres found in the liver after 10 min, unpublished data). These results could be explained by an intrahepatic localization of the doxorubicin-loaded nanospheres in the Küpffer cells, followed by a slow intracytoplasmic release of the drug from the carrier probably in the lysosomes having a suitably low pH for the desorption of the drug; DXR could then reach parenchymal cells and be metabolized.

The nanoparticles may act as intrahepatic slow-release systems, giving an increase of the anthracycline concentration in this tissue and a modification of its metabolism kinetic. Considering these observations, an increased activity of DXR in the liver can be expected and DXR-loaded nanospheres could be perhaps of interest for hepatocarcinoma treatments.

One hour after the injection of DXR-loaded nanoparticles, the concentrations of DXR and DXone in the kidneys are respectively 1.3- and 1.7-fold higher whereas DXol level is 2.5-fold lower, compared to the free injected anthracycline. The concentrations of DXol in the kidneys decrease from 1 to 96 h for the free form, corresponding to a classical elimination kinetic, and inversely for the bound DXR the tissue levels increase from 1 to 24 h (Table 3). This inversed tissue levels profile, observed for DXol in the kidneys, could be also a consequence of the perturbation in the hepatic metabolism kinetic of the parent drug.

For the spleen, the lungs, and the heart, no significant tissue concentration differences were found between the two administered preparations during the experience time (Fig. 4).

TABLE 3

Tissue levels of doxorubicin and metabolites in rabbits, after a 1.54 mg/kg intravenous injection of: (a) free doxorubicin; and (b) DXR-loaded nanoparticles, without surfactant

N.D. = not determined.

Tissue concentrations (μg DXR equivalents/g of organ fresh weight)		Liver			Kidneys			
		1 h	6 h	24 h	1 h	6 h	24 h	96 h
Doxorubicinol	(a)	4.82	0.59	0.41	18.89	14.74	6.01	3.03
	(b)	2.18	0.68	0.47	7.48	11.83	12.46	2.52
Doxorubicin	(a)	2.14	0.81	0.66	14.22	8.91	5.79	1.33
	(b)	3.42	0.85	0.60	18.53	7.99	5.56	0.90
Doxorubicinone	(a)	3.99	N.D.	N.D.	4.36	0.53	N.D.	N.D.
	(b)	1.95	0.45	N.D.	7.54	0.63	0.25	N.D.

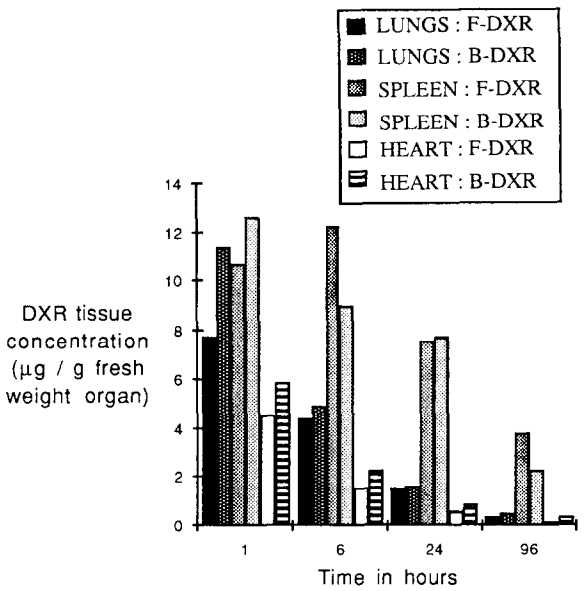

Fig. 4. Concentration–time profiles of doxorubicin in lungs, spleen and heart, after a 1.54 mg/kg intravenous injection of free and bound DXR in the rabbit.

The differences particularly observed 1 h after the administration of F-DXR and B-DXR could be more important for shorter times after injection. Indeed, the distribution half-life of the nanoparticles is very short, about 3 or 4 min (data not shown) and some authors (Couvreur et al., 1980) have shown for another cytostatic drug, vinblastine, adsorbed on polyethylcyanoacrylate nanoparticles that modifications of the drug distribution observed 0.5 h after injection of nanoparticle-adsorbed vinblastine disappeared after 3 h.

Although no significant reduction of DXR levels was determined in the heart for the injected B-DXR, a reduced cardiac toxicity could be hoped for; indeed Forssen and Tokes (1983), using liposome entrapment as a means for overcoming the cardiac toxicity of doxorubicin, have demonstrated that the complete exclusion of DXR from the cardiac tissue is not essential for the elimination of chronic toxicity. The reduced toxicity could be caused by a different cardiac subcellular distribution of drugs linked to carriers to sites not involved in toxicity, and also by a slow release of the drugs from the nanoparticles as seen before for anthracycline–DNA complex (Ohnuma et al., 1975).

Conclusions

The pharmacokinetic and tissue distribution modifications observed after intravenous injection of bound-doxorubicin in rabbits, by means of anthracycline targeting using polymethacrylic nanoparticles as carriers, could be hoped to reduce the toxicity and side-effects of DXR and to increase its therapeutic activity in cancer chemotherapy, especially in hepatoma treatment. Doxorubicin-loaded nanoparticles are actually administered intravenously to patients with hepatocarcinoma, for preliminary clinical assays, and the first results are encouraging, resulting in a reduction of the DXR toxic effects (nausea, vomiting, cardiomyopathy, medullar depression) and in an apparent higher therapeutic activity.

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