Cancer Chemother Pharmacol (1990) 26: 13–18

Cancer Chemotherapy and Pharmacology

Tissue distribution of doxorubicin associated with polyisohexylcyanoacrylate nanoparticles

Claudette Verdun[1], Francis Brasseur[2], Henri Vranckx[1], Patrick Couvreur[3], and Michel Roland[4]

[1] Sopar S. A., B-1080 Brussels, Belgium
[2] Unité d'Oncologie, Université Catholique de Louvain, B-1200 Brussels, Belgium
[3] Laboratoire de Pharmacie Galénique et Biopharmacie CNRS UA 1218, Université Paris XI, F-92290 Chatenay-Malabry, France
[4] Unité de Pharmacie Galénique, Université Catholique de Louvain, B-1200 Brussels, Belgium

Received 17 February 1989/Accepted 5 August 1989

Summary. The body distribution of i.v. doxorubicin depends mainly on the physicochemical characteristics of the molecule. However, entrapment of that cytostatic drug inside polyalkylcyanoacrylate nanoparticles has been shown to modify its distribution profoundly in the mouse. Polyisohexylcyanoacrylate nanoparticles loaded with [^{14}C]-doxorubicin were studied in comparison with free drug, with emphasis on their distribution pattern in mouse tissue after i.v. administration. An autoradiographic study showed that most of the radioactivity was found in the reticuloendothelial system as soon as a few minutes after i.v. administration of the doxorubicin-loaded nanoparticles. Quantitative determinations by liquid scintillation counting in fresh tissue (spleen, heart, kidneys, liver, lungs, bone marrow) and blood samples confirmed these observations. When the drug was linked to nanoparticles, doxorubicin blood clearance was reduced during the first few minutes after administration, whereas heart and kidney concentrations were substantially decreased. Assays of doxorubicin and doxorubicinol by a specific HPLC analytical method gave results very similar to those obtained by scintillation counting.

Introduction

The toxicity and the efficacy of a drug can be favourably influenced by its association with an intravascular colloidal carrier [1, 3]. Not only is enhanced protection against biological mediums attained, but the tissue distribution or cellular tropism of the molecule carried may be modified according to the intrinsic properties of the carrier itself. Such modifications could be useful for better targeting or for avoiding organs sensitive to the toxic effects of the drug. Moreover, slow drug release from a storage organ could help to enhance the therapeutic index and reduce side effects.

Especially for cytotoxic drugs, improved tissue specificity could be achieved by vectorization. In particular, doxorubicin, which possesses a wide spectrum of activity but is responsible for chronic cardiotoxicity, bone marrow depression and gastrointestinal disorders [5], has been found to be less toxic in mice when associated with biodegradable nanoparticles [3]. Furthermore, the antitumor activity of the drug was noticeably enhanced when it was used in its nanoparticulate form; this effect was observed in both experimental solid and non-solid tumors as well as in experimental metastases.

However, the mechanism by which targeted drugs are more efficient than free drug remains unclear. In particular, information on the pharmacodistribution of doxorubicin-loaded nanoparticles should lead to a better understanding of these pharmacological observations. Therefore, this paper describes the tissue-distribution profile of doxorubicin associated with polyisohexylcyanoacrylate nanoparticles. [^{14}C]-Scintillation counting data were compared with those determined by a specific HPLC method. Data are also given concerning the excretion rate of nanoparticle-bound doxorubicin.

To confirm the importance of a modification in the distribution pattern of doxorubicin, it was necessary to compare the whole-body distribution of the i.v. injected cytostatic both as free drug and in association with the carrier.

Materials and methods

Doxorubicin hydrochloride was a gift of Sicor (Italy). [^{14}C]-Doxorubicin hydrochloride with a specific activity of 56 mCi/mmol was obtained from Amersham (England). Isohexylcyanoacrylate was synthesized by Sopar S. A. (Belgium). Other chemical compounds included pyrogen free dextran (Pharmacia, Sweden), glucose and citric acid (Merck, Germany). The radiosensitive films (Hyperfilm β max) were purchased from Amersham (England). The chemicals used for radiation counting, Carbo-sorb and Permafluor, were obtained from Packard (Packard Downers Grove, USA). Acetonitrile RS for HPLC was obtained from Farmitalia Carlo Erba (Italy). Male NMRI mice weighing between 20 and 25 g were used in all experiments.

The HPLC system used for assays of doxorubicin and its metabolites consisted of an M-45 solvent-delivery system and U6-K universal LC injector fitted with a μ-Bondapak C18 column (3.9 mm × 30 cm) containing 10-μm particles (Waters Associates, USA). Detection of the peaks was carried out with a model LS-4 spectrophotofluorimeter (Perkin-Elmer, USA). The chromatograms were registered and the peak surfaces, integrated on the printer of an M 730 data module (Waters Associates, USA).

Offprint requests to: H. Vranckx

Radioactivity assays

Preparation of [^{14}C]-doxorubicin nanoparticles. Nanoparticles were obtained by anionic polymerization of isohexylcyanoacrylate monomer [4, 13]. Briefly, 25 μCi (preparation A) or 12.5 μCi (preparation B) radioactive doxorubicin hydrochloride was added together with 1.875 mg non-labelled doxorubicin to a 2.5-ml aqueous solution of 5% glucose, 1% dextran 70 and 0.06% citric acid. Under mechanical stirring, 25 mg isohexylcyanoacrylate monomer was then dropped into the medium and allowed to polymerize. After 5 h polymerization, nanoparticles were obtained.

Size determination was carried out using a laser light-scattering method (Nano Sizer, Coulter). After ultracentrifugation of the nanoparticle sample and measurement of the radioactivity, the level of drug binding was estimated by scintillation counting in sediment dissolved in acetonitrile (bound drug) and in supernatant (free drug).

Injection procedures. Radioactive suspensions were injected into a caudal vein of mice: 3 μCi for whole-body autoradiography (0.3 ml preparation A) and 1 μCi for quantitative determinations (0.2 ml preparation B).

Whole-body autoradiography. This procedure was carried out according to the method previously described by Ullberg [12]. After the injection of radioactive doxorubicin nanoparticles, mice were killed at various intervals (5 and 30 min, 1, 2, 4, 8 and 24 h), frozen by immersion in liquid nitrogen and embedded in an 1.5% aqueous solution of sodium carboxymethylcellulose. After storage at −30° C for 48 h, mice were sectioned with a cryomat (Cryotome 1714; Leitz, Germany); saggital sections 40 μm thick were taken on adhesive tape and dried in a cold room. After cryodessication (48 h) in a deep-freezer, all sections were pressed against radiosensitive film for 1–3 weeks. The dark areas on the developed film corresponded to high radioactive concentrations.

Tissue radioactivity assays and cumulative excretion determination. After the i.v. injection, animals (eight mice/group) were sacrificed at different intervals (5 and 30 min, 1, 2, 4, 8 and 24 h, 3 and 5 or 8 days). Various organs, including the heart, liver, spleen, lungs and kidneys, and blood were removed and weighed. The bone marrow was extracted from both femurs of all animals and pooled before radioactivity determination. Tissue samples were treated in an oxidizer (Packard, Packard Downers Grove, USA) following the method of Peterson [8]. Briefly, ^{14}C was converted in carbon dioxide after combustion in an atmosphere with a continuous flow of oxygen. Carbon dioxide was trapped in the scintillation fluid and radioactivity was measured by scintillation counting (Minibeta; LKB Wallac, Finland).

Urine and faeces were collected in two groups of eight mice kept in metabolic cages, and radioactivity was determined 1, 2, 3, 5 or 8 days after i.v. administration. These samples were treated in the same way as tissue samples. Drug concentration in each tissue was expressed as the percentage of the injected dose in the whole, fresh organ.

HPLC assays

The HPLC methodology was adapted from Pierce's previously described method for plasma samples [9] and extraction procedures were carried out according to Rolland [11].

Preparation of Doxorubicin nanoparticles. Conditions of polymerization were the same as those mentioned above for [^{14}C]-doxorubicin-loaded nanoparticles, with identical drug and polymer concentrations. Determinations of size and the level of drug binding were also carried out.

Injection procedure. Doxorubicin was given as a bolus injection into a caudal vein at a dose of 7.5 mg/kg (0.2 ml suspension).

Tissue sampling. Animals (ten mice) were sacrificed in 1 h after i.v. injection, and major organs (heart, liver, spleen, lungs, kidneys) were removed and weighed before lyophilization. Lyophilized organs were weighed again, grinded and stored at −20° C until assayed by HPLC. Results were expressed as the percentage of the injected dose in the whole, fresh organ.

Extraction procedure. Distilled water (2 ml) was added to 100 mg pulverized organ. The suspension was mechanically stirred for 3 min at 20,000 rpm and homogenized for 5 min at 150 W by sonication using an Ultrasonics sonicator (Ultrasonics Ltd, England). In all, 100 μl solution containing 20% trichloroacetic acid in a mixture of acetonitrile-water (40/60, v/v) and desipramine (10 μg/ml) and 10 μl daunorubicin solution (200 μg/ml) were added to 100 μl of this suspension.

Samples were stirred for 30 s (Paramix II; Julabo Labortechnik GmbH, Germany) and centrifugated at 12,000 rpm for 5 min. Fluid supernatant (50 μl) was introduced into a tronconic tube in which a 150-μl mixture of 0.2 *M* sodium acetate in methanol was evaporated.

HPLC assay procedure. To 50 μl supernatant fluid was added 100 μl mobile phase; an aliquot of this solution was injected into the chromatograph. The mobile phase, filtered on a 0.45-μm Durapore membrane (Millipore, USA), consisted of acetonitrile and 0.01 *M* phosphoric acid (40/60, v/v); it was used at a flow rate of 1.5 ml/min. Excitation and emission wavelengths were 488 and 590 nm, respectively.

Method specificity. Retention times of doxorubicinol, doxorubicin and daunorubicin were 1.6, 3 and 4.8 min, respectively. It was demonstrated that the extraction method did not produce metabolites of doxorubicin: after extraction from an aqueous phase containing doxorubicin and daunorubicin, no degradation compounds could be detected. On the other hand, doxorubicinol was detected in organs of treated animals (Fig. 1).

Method sensitivity. The detection limit of doxorubicin was defined as a signal-to-noise ratio of 3 : 1. This ratio was achieved at 0.3 ng doxorubicin, and the limit quantitation for liver was 0.6 μg/g fresh organ.

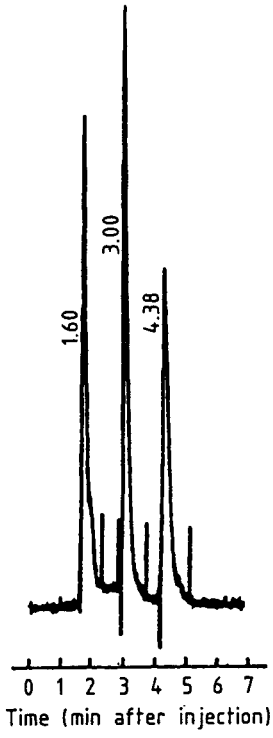

Fig. 1. Chromatogram of a mouse liver specimen, showing the separation of doxorubicinol, doxorubicin and daunorubicin by HPLC. At a flow rate of 1.5 ml/min, the following t_R were obtained: doxorubicinol, 1.6; doxorubicin, 3.0; and daunorubicin, 4.38

Statistics

In experiments where multiple measurements were made (dose-response relationship), differences between groups were tested for significance using a two-way analysis of variance by the SAS procedure ANOVA (*SAS User's Guide*, "Statistics"); the major and crossed effects were tested. The ANOVA test accommodates terms such as subjects, organ and formulation as well as the residual error. If the probability of the null hypothesis was not sufficiently strong, it was rejected. The factor was declared significant if the probability of the null hypothesis H_0 was $<5\%$ and highly significant if H_0 was $<1\%$.

Results

Characteristics of polyisohexylcyanoacrylate nanoparticle suspensions

The average diameter of particles used for distribution studies (loaded with [^{14}C]- or non-labelled doxorubicin) was 260 ($\pm$15) nm. In each case, 95% of the doxorubicin initially dissolved in the polymerization medium (750 µg/ml) was firmly bound to the carrier (corresponding to 71.25 mg drug/g polymer).

Autoradiographic study

At 5 min after i.v. administration of [^{14}C]-doxorubicin-loaded nanoparticles, radioactivity mainly concentrated in the liver, lungs and spleen (Fig. 2). After 4 h, no important modification was observed in both distribution and radioactive concentration (Fig. 3). Except in the liver and spleen, where it remained extremely marked, radioactivity was noticeably reduced in all organs 24 h after administration (Fig. 4).

Fig. 2. Whole-body autoradiography of a mouse 5 min after i.v. administration of [^{14}C]-doxorubicin nanoparticles

Fig. 3. Whole-body autoradiography of a mouse 4 h after i.v. administration of [^{14}C]-doxorubicin nanoparticles

Fig. 4. Whole-body autoradiography of a mouse 24 h after i.v. administration of [^{14}C]-doxorubicin nanoparticles

Quantitative tissue distribution

Autoradiographic data were confirmed by tissue scintillation counting. As shown in Table 1, radioactivity was mainly localized in the liver; indeed, as soon as 5 min after i.v. administration, 75% of the injected dose was found in that organ. At longer intervals, the liver also retained most of the drug, although the radioactivity gradually decreased in that organ; after 8 days, only small amounts of labelled compound were still present. In the same way, but to a lesser extent, the spleen and lungs retained more cytostatic agent when it was associated with the carrier.

On the other hand, radioactivity in the heart and kidneys was lower than free-drug levels when doxorubicin was attached to nanoparticles. However, this diminution

Table 1. Tissue distribution of free and nanoparticle-bound [^{14}C]-doxorubicin[a]

		5 min	30 min	1 h	2 h	4 h	8 h	24 h	3 days	8 days
Liver	Free DOX	30.7 ±5.1	34.8 ±8.7	33.3 ±3.4	34.9 ±3.1	17.0 ±1.7	13.6 ±0.6	–	–	–
	IHCA DOX	74.7 ±2.9	62.1 ±2.2	71.7 ±3.9	70.8 ±2.5	63.7 ±4.5	62.2 ±5.4	57.7 ±2.69	8.6 ±0.75	2.7 ±0.42
Heart	Free DOX	0.60±0.12	0.75±0.04	0.54±0.05	0.59±0.04	0.31±0.02	0.28±0.01	–	–	–
	IHCA DOX	0.50±0.03	0.23±0.03	0.21±0.05	0.18±0.01	0.17±0.04	0.15±0.03	0.14±0.02	0.09±0.02	0.02[b]
Lungs	Free DOX	1.25±0.24	1.55±0.08	1.09±0.21	1.43±0.12	0.76±0.06	0.94±0.07	–	–	–
	IHCA DOX	3.37±1.04	5.36±0.47	3.70±1.18	5.49±1.12	1.63±0.24	1.13±0.19	1.4 ±0.17	1.2 ±0.52	0.1 ±0.01
Spleen	Free DOX	0.50±0.12	0.69±0.07	0.62±0.07	1.05±0.06	0.54±0.06	0.65±0.05	–	–	–
	IHCA DOX	1.62±0.14	1.78±0.35	2.49±0.78	2.05±0.68	2.90±0.59	2.40±0.19	1.4 ±0.37	2.8 ±0.74	2.8 ±0.89
Kidneys	Free DOX	2.97±0.47	3.57±0.24	2.46±0.29	3.42±0.16	2.60±0.16	2.44±0.11	–	–	–
	IHCA DOX	0.88±0.07	0.47±0.05	0.55±0.11	0.51±0.02	0.41±0.04	0.27±0.03	0.48±0.04	0.38±0.04	0.09±0.01
Blood	Free DOX	2.84±0.43	1.60±0.14	1.27±0.06	1.46±0.06	0.88±0.06	0.69±0.08	–	–	–
	IHCA DOX	10.00±1.11	2.22±0.75	1.41±0.26	1.09±0.14	0.25±0.03	0.14±0.01	0.15±0.01	0.16±0.01	0.13±0.02

[a] Tissue distribution is expressed as the percentage of injected drug in each organ; plasma concentrations are expressed in µg/ml. All data represent the means ± SEM

[b] SEM <0.01

DOX, doxorubicin; IHCA DOX, doxorubicin-loaded polyisohexylcyanoacrylate nanoparticles

of heart impregnation was not observed for intervals as short as 5 min after administration (Table 1). This was probably due to contamination of the heart by circulating blood, which contained very high levels of radioactivity for short intervals after administration. The lower levels of doxorubicin found in the kidneys of mice injected with nanoparticles could be explained by reduced urinary excretion (Table 2). In contrast, faecal excretion was only slightly modified. This observation is in accordance with the affinity of nanoparticles for the liver, which probably represents the major metabolization site of the carrier [6]. Blood clearance was rapid in both cases, the main difference appearing during the first few minutes after administration.

Bone marrow assays showed delayed capture when the cytostatic agent was associated with nanoparticles (Table 3). The statistical ANOVA analysis (Table 4) showed that for each organ examined, highly significant differences in radioactivity were found after i. v. administration of nanoparticle-bound [^{14}C]-doxorubicin in comparison with the free, labelled drug. Except in the spleen, significant variations in drug concentration as a function of time were also observed. Moreover, in the heart, lungs and blood, a crossed effect was detected: the formulation influenced the pharmacokinetic profile of the drug (Fig. 5).

HPLC assays confirmed the results obtained by ^{14}C scintillation counting (Table 5). For instance, in the liver at 1 h after i. v. administration, 36.5% of the doxorubicin and 32.4% of the doxorubicinol was found, giving a total of 68.9% for the two anthracyclines (71.7% was determined by scintillation counting). Excellent correlations between the results of HPLC and scintillation counting were also obtained in other tissues.

Discussion

After i. v. injection, the tissue distribution of doxorubicin was dramatically modified when the drug was linked to polyisohexylcyanoacrylate nanoparticles. For instance, Fig. 6 shows the comparative tissue-distribution profile of doxorubicin 1 h after administration.

Table 2. Urinary and faecal excretion of [^{14}C]-doxorubicin as free drug and associated with polyisohexylcyanoacrylate nanoparticles

		1 day	2 days	3 days	5 days	8 days
Urine	Free DOX	11.22	13.31	13.59	14.16	–
	IHCA DOX	2.35	2.50	3.75	–	4.30
Faeces	Free DOX	19.85	48.77	64.50	65.90	–
	IHCA DOX	13.82	32.24	54.20	–	80.74

Data are expressed as a percentage of the injected dose

Table 3. Bone marrow content of [^{14}C]-doxorubicin as free drug and associated with polyisohexylcyanoacrylate nanoparticles

	5 min	30 min	1 h	2 h	4 h	8 h	24 h	8 days
Free DOX	0.85	1.20	0.83	0.91	0.34	0.38	–	–
IHCA DOX	0.08	0.08	0.02	0.06	0.09	0.36	0.24	0.42

Data are expressed as a percentage of the injected dose per gram of wet tissue

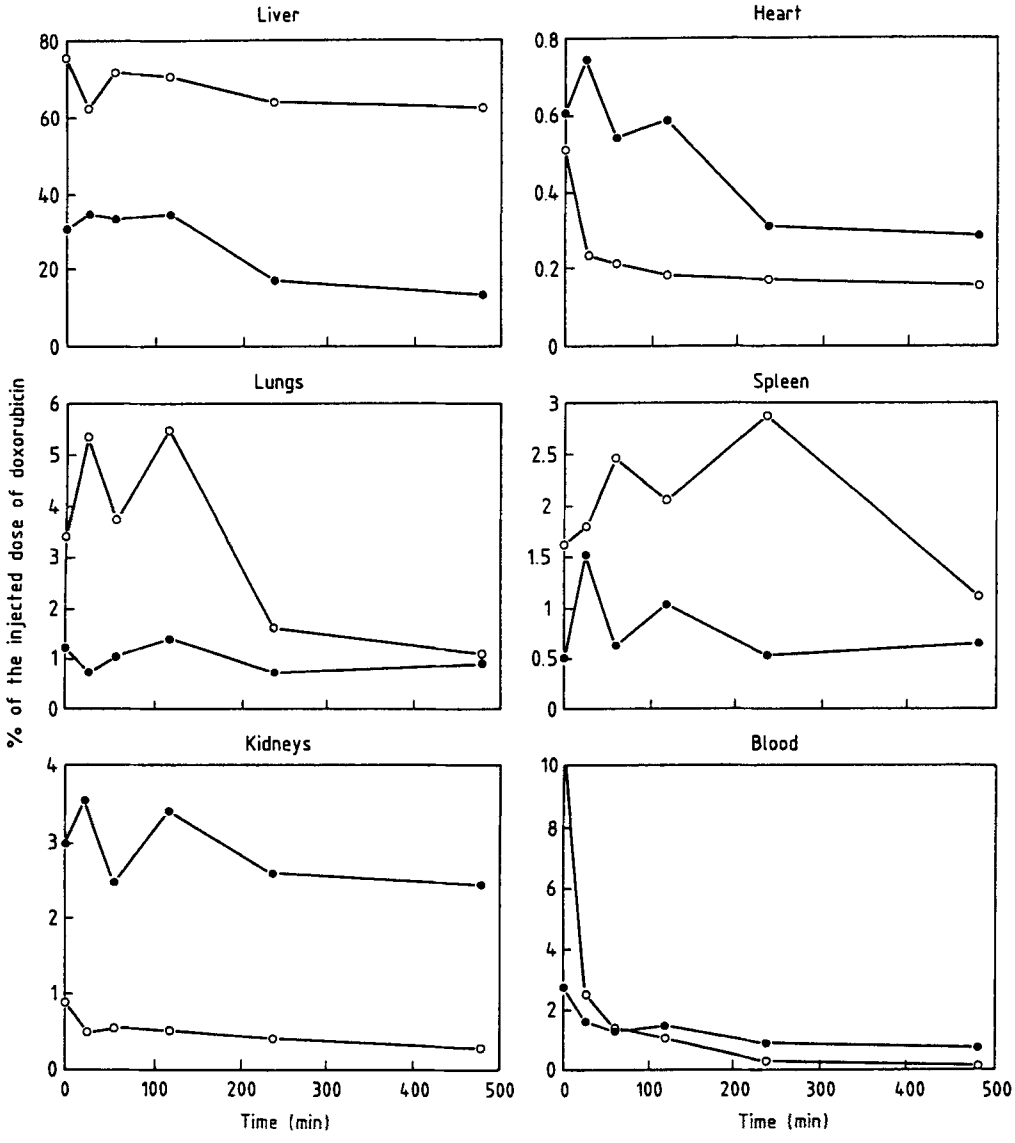

Fig. 5. Tissue concentrations of free (●) and nanoparticle-bound [^{14}C]-doxorubicin (○), expressed as the percentage of the injected drug in each organ, and blood concentration (μg/ml)

Vectorized doxorubicin mainly concentrated in the liver and lungs, as well as in the spleen. This leads to the possibility of delivering more effective doses of drug to organs that are common sites of metastasic disease.

Table 4. Statistical evaluation of the influence of time and formulation (nanoparticle-bound and free drug) on doxorubicin concentrations in several organs according to the two-way variance analysis ANOVA

Organ	Time	Formulation	Time × formulation
Liver	**	**	7%
Heart	**	**	**
Lungs	**	**	**
Spleen	35%	**	25%
Kidneys	**	**	9%
Blood	**	**	**

* Significant difference; ** Highly significant difference

Moreover, the increased efficacy of doxorubicin associated with nanoparticles has been observed in experimental liver metastases in mice [2].

Liver concentrations of radioactivity due to nanoparticle-bound doxorubicin remained at a high level during the 1st day. As a consequence of faecal excretion, liver concentrations gradually decreased; this reduction occurred even more rapidly during days 2 and 3. After 8 days, only about 3% of the labelled cytostatic drug could be detected. Previously published observations [7] have shown that drug-release kinetics is very similar to that of bioerosion of the polymers. Our data are therefore consistent with a limited risk of polymer accumulation when several cures are needed. Likewise, lung impregnation progressively became similar for free and vectorized drug at 8 h after treatment.

Previous studies using doxorubicin-loaded albumin microcapsules have shown that the in vivo distribution of the drug was modified in terms of a higher capture by the lung tissue [14]. This particular distribution profile could

Table 5. Tissue distribution of doxorubicin and doxorubicinol 1 h after i. v. injection of nanoparticle-bound doxorubicin according to HPLC assays

Organs	Doxo-rubicin	Doxo-rubicinol	Total
Liver	36.5	32.3	68.9
Spleen	1.3	0.9	2.2
Lungs	1.1	1.7	2.8
Kidneys	0.24	0.13	0.37
Heart	0.03	0.17	0.20

Data are expressed as the percentage of injected drug in the whole, fresh organ

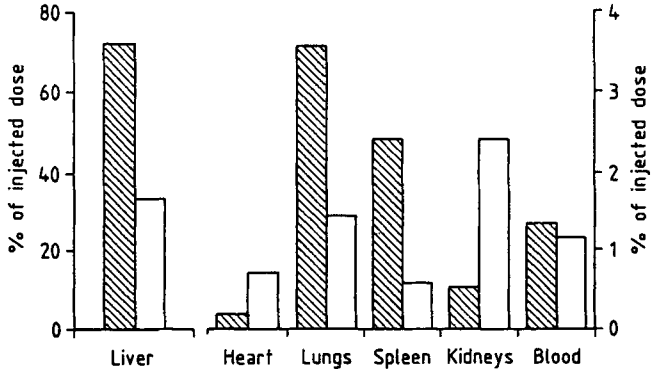

Fig. 6. Comparative organ and blood distribution of [^{14}C]-doxorubicin in mice 1 h after injection of either free or vectorized drug, expressed as a percentage of the injected drug. ▨ IHCA DOX; □ free DOX

be explained by the possible embolization of lung capillaries due to the size of the particles used (15 μm). In the case of doxorubicin-loaded polyisohexylcyanoacrylate nanoparticles, the smaller size (260 nm) of the carrier avoids lung embolization and enables, in contrast, a preferential capture by liver macrophages [6]. This profile is similar to previous observations made with doxorubicin entrapped in cardiolipin liposomes, for which terminal half-lives in the liver and spleen were 15- and 2.3-fold higher, respectively, than those measured for the free drug [10].

Moreover, the significant reduction in radioactive cytostatic concentrations obtained in the heart and kidneys when the drug was associated to polyisohexylcyanoacrylate nanoparticles opens interesting perspectives for future clinical applications. These data illustrate more particularly the possibility of avoiding high cardiac levels of doxorubicin by linkage to nanoparticles, suggesting the potential of this approach for reducing the heart toxicity of doxorubicin.

Acknowledgements. The authors are grateful to Dr. J. Cumps for his statistical work and interpretation and are indebted to J. P. Vandiest for the drawings and photographic work. The technical assistance of Mrs. P. S. Dolotabadi is greatly appreciated. Typing of the manuscript was carried out by Dr. Vranckx. This study was supported by Sopar Pharma, Belgium.

References

1. Brasseur F, Couvreur P, Kante B, Passau L, Roland M, Deckers C, Speiser P (1980) Actinomycin D adsorbed on polymethylcyanoacrylate nanoparticles increased efficiency against an experimental tumor. Eur J Cancer 16: 1441
2. Chianilkulchai N, Driouich Z, Benoit JP, Gantier JC, Couvreur P (1989) Doxorubicin-loaded nanoparticles: increased efficiency in murine hepatic metastases. Cancer Drug Deliv (in press)
3. Couvreur P, Kante B, Grislain L, Roland M, Speiser P (1982) Toxicity of polyalkylcyanoacrylate nanoparticles: II. Doxorubicin-loaded nanoparticles. J Pharm Sci 71: 790
4. Donnelly EF, Johnston DS, Pepper DC, Dunn DJ (1977) Ionic and zwitterionic polymerization of *n*-alkyl-2-cyanoalkylacrylates (Letter to the editor). Polymer 15: 399
5. Lefrak E, Pitha J, Rosenheim S, Gottlieb J (1973) A clinicopathologic analysis of Adriamycin cardiotoxicity. Cancer 32: 302
6. Lenaerts V, Nagelkerke JF, Van Berkel TJC, Couvreur P, Grislain L, Roland M, Speiser P (1984) In vivo uptake of polyisobutylcyanoacrylate nanoparticles by liver Kupffer, endothelial and parenchymal cells. J Pharm Sci 73: 980
7. Lenaerts V, Couvreur P, Christiaens-Leyh D, Joiris E, Roland M, Rollman B, Speiser P (1984) Degradation of poly(isobutylcyanoacrylate) nanoparticles. Biomaterials 5: 65
8. Peterson J (1969) A carbon dioxide collection accessory for the rapid combustion apparatus for preparation of biological samples for liquid scintillation analysis. Anal Biochem 31: 204
9. Pierce RN, Jatlow PI (1979) Measurement of Adriamycin (doxorubicin) and its metabolites in human plasma using reversed-phase high-performance liquid chromatography and fluorescence detection. J Chromatogr 164: 471
10. Rahman A, Carmichael D, Harris M, Roh JK (1986) Comparative pharmacokinetics of free doxorubicin entrapped in cardiolopin liposomes. Cancer Res 46: 2295
11. Rolland A (1987) Mise au point et application de nanosphères à base de copolymères méthacryliques. Intérêts pour la vectorisation d'agents cytostatiques (anthracyclines). Ph.D. Thesis, University of Rennes I
12. Ullberg S (1954) Studies on the distribution and fate of S^{35}-labelled benzylpenicillin in the body. Acta Radiol 118: 1
13. Verdun C, Couvreur P, Vranckx H, Lenaerts V, Roland M (1986) Development of a nanoparticle controlled-release formulation for human use. J Controlled Release 3: 205
14. Wilmott N, Cummings J, Stuart JBF, Florence AT (1985) Adriamycin-loaded albumin microspheres: preparation, in vivo distribution and release in the rat. Biopharm Drug Dispos 6: 91