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Clinical pharmacokinetics of doxorubicin in hepatoma patients after a single intravenous injection of free or nanoparticle-bound anthracycline

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Summary

In preliminary clinical trials, following a single intravenous injection of doxorubicin-loaded nanospheres into hepatoma patients, plasma concentrations of the drug have been determined and compared to those obtained after administration of free doxorubicin. After an efficient extraction of doxorubicin (DXR) and daunorubicin (DNR) (as internal standard) from plasma (87–88%), the anthracyclines were separated by reversed-phase high-performance liquid chromatography within 12 min. Detection limit was 5 ng of DXR/ml of plasma and peak-height ratio of DXR versus DNR showed a linear correlation for concentrations ranging from 5 to 1000 ng/ml. After intravenous administration of nanoparticle-bound doxorubicin into 4 patients with hepatocellular carcinoma, a prolonged elevation in DXR plasma levels was observed compared to the profile obtained after injection of free DXR. In addition, the administration of doxorubicin-loaded nanospheres led to a reduction of the distribution volume and of the total clearance of DXR, probably owing to the passive drug targeting to the liver.

Introduction

Doxorubicin (adriamycin) is a potent cytostatic drug active against a wide spectrum of human malignancies, including hepatocellular carcinoma (Olweny et al., 1975; Vogel et al., 1977; Ihde et al., 1977; Bern et al., 1978; Chan et al., 1980; Chlebowski et al., 1984). However, the therapeutic application of this anthracycline antibiotic is limited by its side-effects, mainly dose-dependent myelosuppression and chronic cardiotoxicity (Henderson and Frei, 1980; Ferrans, 1983; Haq et al., 1985; Speth et al., 1988). In an attempt to increase the therapeutic index of doxorubicin (DXR), drug-carriers have been developed such as liposomes (Forssen and Tokes, 1981; Van Hoesel et al., 1984; Mayhew et al., 1987), niosomes (Rogerson et al., 1987, 1988), microspheres (Widder et al., 1980; Pfeifle et al., 1986; Gupta et al., 1987, 1988; Willmott and Harrison, 1988; McArdle et al., 1988; Kerr et al., 1988), DNA

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(Kummen et al., 1978; Baurain et al., 1982; Gunven et al., 1986) and monoclonal antibodies (Sinkule et al., 1987; Rowland, 1987; Rolland and Bourel, 1989). The main goals of these site-specific drug delivery systems are as follows:

- increased selectivity to the cancer cells without any loss of DXR efficacy;
- reduced DXR toxicity for the normal tissues, mainly for the bone marrow and cardiac muscle;
- DXR protection from premature inactivation during the transport to the cancer cells;
- DXR retention at the site of action and controlled release from the carrier; and
- possibility of modifying the treatment schedules, e.g. reducing the administered doses.

Recently, polymethacrylic nanoparticles have been proposed as a potential site-specific drug delivery system (Rolland et al., 1986a, b and c). Although these particles are slowly biodegraded, their acute toxicity in mice ($LD_{50} \simeq 720$ mg/kg) and subacute toxicity in rabbits (Rolland, unpublished results) did not point out any figure susceptible to hinder the use of polymethacrylic nanospheres in human medicine with a monthly injection protocol. A high loading of DXR to polymethacrylic nanoparticles was previously demonstrated (up to a concentration of 1.7 mM) (Rolland et al., 1986c) and DXR antitumor activity in U-937 cells was proved to be enhanced when the anthracycline was first bound to the nanospheres, as compared to the free drug (Astier et al., 1988).

In addition, pharmacokinetics and biodistribution of nanoparticle-bound DXR were shown to be modified after a single intravenous injection into rabbits (Rolland, 1988). Since a rapid and intense particle uptake by the mononuclear phagocyte system (MPS), mainly by the liver, was observed (Rolland et al., 1989) the nanospheres were thought to act as an intrahepatic reservoir slowly releasing DXR, as also suggested earlier with liposomes (Mayhew et al., 1983; Van Hoesel et al., 1984; Gabizon et al., 1985). As observed previously in rabbits, the passive targeting of DXR to the liver not only increased its hepatic concentration but also induced a modification of its metabolism kinetics. Therefore, with the aim of improving the therapeutic index of DXR, 4 patients with hepatocellular carcinoma, who had a histological diagnosis, evidence of surgical non-resectability and a poor prognosis, received intravenous injections of DXR-loaded nanospheres.

The present study presents the DXR plasma concentrations, quantified by reversed-phase high-performance liquid chromatography (HPLC), after the first intravenous injection of either free or nanoparticle-bound DXR. The pharmacokinetic parameters, estimated according to a two-compartment open model, are then discussed.

Materials and Methods

Chemicals

Doxorubicin (Adriblastine) was purchased from Roger Bellon Lab. (France) and daunorubicin (Cerubidine) from Specia Lab. (France).

The methacrylic monomers were obtained from Merck (France) and purified before use by distillation as described previously (Rolland et al., 1986a).

All other chemicals were of analytical grade.

Preparation of free and nanoparticle-bound doxorubicin

Injectable doxorubicin solution (free DXR) was obtained by dissolution of the lyophilized powder in sterile saline to a concentration of 1 mg/ml.

Polymethacrylic nanoparticles were prepared by aqueous emulsion polymerization of a 2.5% (v/v) mixture of monomers (methyl methacrylate; 2-hydroxypropyl methacrylate; methacrylic acid; ethylene glycol dimethacrylate) as previously described (Rolland et al., 1986a and b).

After filtration (glass filter 10–20 μm), the nanoparticle suspension was purified by dialysis (Hollow Fiber Dialyser GF 180M, Gambro, F.R.G.) and the absence of remaining monomers was verified by HPLC (Rolland et al., 1986c). The suspension was adjusted to pH 8.4 with 0.01 M sodium acetate and 1 M sodium hydroxide, then sterilized by autoclaving (Sano Clav, Bioblock, France) at 121°C for 20 min. Biological (Attest, Medical Products Division/3M, U.S.A.) and chemical indicators (Stericontrol, Eschmann, France) were used to valid the sterilization pro-

cess, then sterility tests were performed for each lot.

DXR solution was prepared with sterile water to a concentration of 10 mg/ml, then under sterile conditions 3 ml of the DXR solution was added dropwise with mixing to 27 ml of the sterilized nanosphere suspension. The preparation of nanoparticle-bound doxorubicin (1 mg/ml) was left at 4° C overnight in order to reach an optimal drug binding.

The nanoparticle size was measured using a Coulter Nano-Sizer (Coultronics, France). The in vitro release of DXR from the nanospheres was studied by dialysing either free or bound DXR in phosphate-buffered saline (PBS) at pH 7.45 and by measuring the remaining DXR in the dialysis bag at various times by HPLC.

Drug administration

Sterile preparations of free or bound doxorubicin were intravenously injected into 4 hepatoma patients at doses of either 20 or 30 mg of drug.

Heparinized blood samples were collected at various time intervals after the injection: 0.5, 1, 2, 3, 6, 12 and 24 h. They were then centrifuged and plasma samples were collected and kept at −20° C until analysis.

Extraction method

0.1 ml of internal standard (aqueous solution of DNR at 1 μg/ml) and 0.25 ml of 0.5 M Tris buffer, pH 8.6, were added to 0.5 ml of plasma sample. 4 ml of ethyle acetate was mixed with the precedent solution for 4 min and after centrifugation for 5 min at 4000 rpm the upper organic phase was collected in mini-vials (Interchim, France). 100 μl of 0.05 M sulphuric acid was mixed with the organic phase for 2 min, and after centrifugation for 5 min at 3000 rpm 100 μl of the aqueous phase was quickly transferred into a tube in which 100 μl of a 0.2 M methanolic solution of sodium acetate had been previously dried. The pH of the extract was then suitable for injection into the HPLC system.

Calibration curves were prepared by spiking blank human plasma with DXR at concentrations ranging from 5 to 1000 ng/ml, then the extraction technique described above was applied.

DXR analysis by HPLC

Although several analytical methods have been described for the quantitative determination of anthracyclines, such as doxorubicin, in plasma (Bachur et al., 1976; Israel et al., 1978; Eksborg et al., 1978; Pierce and Jatlow, 1979; Baurain et al., 1979; Averbuch et al., 1981; Robert et al., 1982; Aszalos, 1984; Van Lancker et al., 1986), HPLC after an appropriate extraction procedure seems the most suitable technique.

In the present work, 40 μl of the aqueous extract was injected into the reversed-phase HPLC, consisting of a Wisp 710 B automatic injector, a model 6000 A constant flow-pump and a 254 nm fixed-wavelength detector model 240 (Millipore-Waters, France). The chromatographic column (μBondapak C18 column, Millipore-Waters, France) was thermostated in a water-bath at 30° C. The mobile phase was composed of methanol, 0.01 M aqueous solution of sodium acetate, acetic acid (70 : 30 : 1) 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 (DXR/DNR).

Results and Discussion

Doxorubicin-loaded nanospheres

The polymethacrylic nanoparticle size was 250 nm (polydispersity index = 0) and it was increased to about 300 nm (polydispersity index = 1) after sterilisation and DXR binding (other physicochemical and biological characteristics of the polymethacrylic nanospheres have been previously described by Rolland et al., 1986c and d; Rolland et al., 1987).

The pH of the sterile suspension of doxorubicin-loaded nanospheres was 8.2 leading to a DXR binding yield of 96%.

The release of DXR from the nanoparticles was very slow in PBS, pH 7.45, with a half-life estimated at about 4 days (Fig. 1). As discussed elsewhere (Astier et al., 1988), it seems that DXR adsorption onto the nanospheres is not the only mechanism involved in the anthracycline binding, ionic binding likely being the essential phenomenon. Thus, the DXR–nanoparticle binding and

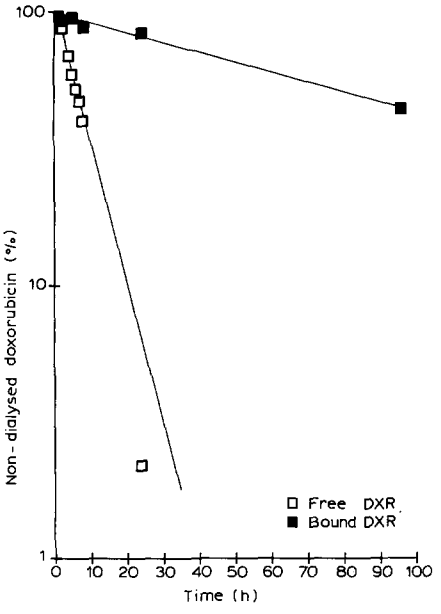

Fig. 1. Dialysis kinetic of free and nanoparticle-bound doxorubicin in PBS, pH 7.45.

release were found to be highly pH-dependent; the higher pH the better the stability.

Dosage of doxorubicin in plasma

For an efficient extraction of doxorubicin and

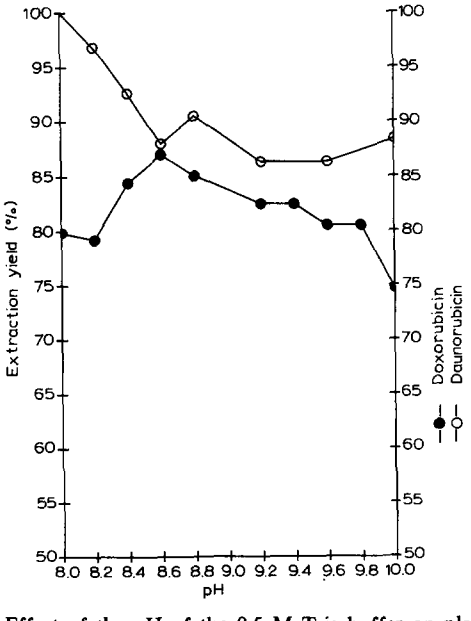

Fig. 2. Effect of the pH of the 0.5 M Tris buffer on plasma extraction efficiencies of DXR and DNR.

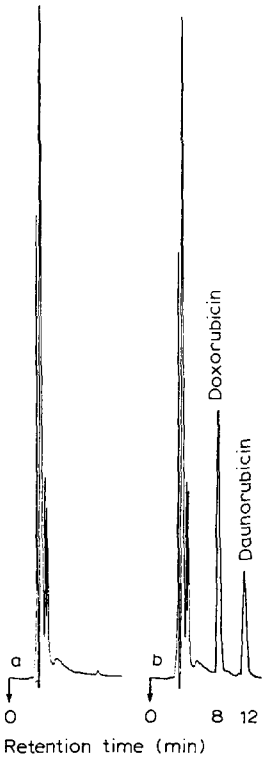

Fig. 3. HPLC separation profile of doxorubicin and daunorubicin (internal standard): chromatograms of (a) extracted blank plasma, (b) plasma spiked with DXR and DNR. (The extraction and chromatographic procedures are described in Materials and Methods.)

daunorubicin, pH of the 0.5 M Tris buffer ranging from 8 to 10 were analysed and a pH value of 8.6 was chosen since the recoveries of the drugs in plasma were 87% and 88% for DXR and DNR, respectively (Fig. 2).

The influence of the alcalinisation of plasma on the extraction yield of doxorubicin and daunorubicin had also been previously reported by other authors (Eksborg, 1978a and b; Eksborg et al., 1978).

The specificity of the analytical method is shown in Fig. 3; the retention times were 8 min for DXR and 12 min for DNR.

Linear calibration curves were obtained up to 1000 ng of DXR/ml of plasma, with correlation coefficients better than 0.999 (Fig. 4).

The lowest detectable amount of DXR was 5 ng of anthracycline/ml of plasma. This detection

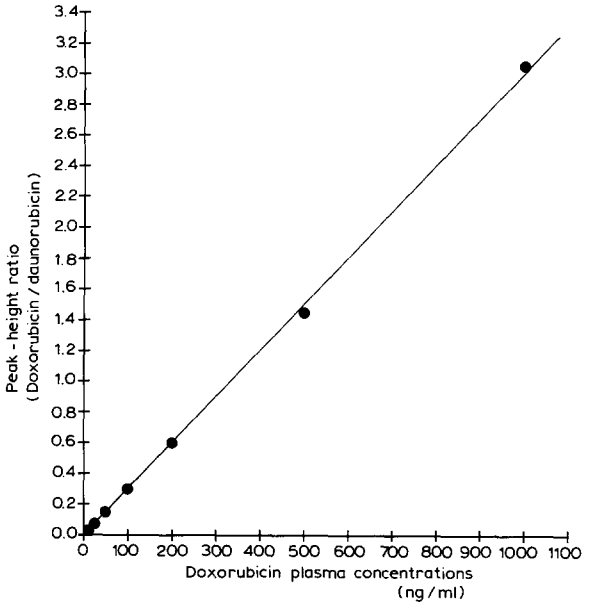

Fig. 4. Mean calibration curve of within-day values ($n = 10$) of DXR plasma concentrations, with daunorubicin as internal standard.

limit could probably be improved using a fluorescence detector as suggested elsewhere (Pierce and Jatlow, 1979; Cummings, 1984; Oosterbaan et al., 1984; Dobbs and James, 1987; Maessen et al., 1987). This analytical method was reproducible, showing a within-day coefficient of variation of 1.9% ($n = 10$).

These methods for the plasma extraction and dosage of anthracyclines, such as doxorubicin, are reliable, rapid, reproducible and sensitive and can therefore be applied to pharmacokinetic studies.

Pharmacokinetic results

After a single intravenous injection of either 20 mg of free DXR or 20 or 30 mg of nanoparticle-bound DXR in hepatoma patients, DXR plasma concentrations were determined using the technique described above. The DXR plasma concentration–time profiles shown in Fig. 5 correspond to biphasic curves. They indicate a first rapid distribution phase followed by a slow elimination phase. These bi-exponential curves are in accordance with other profiles obtained after intravenous administration of DXR in patients (Benjamin et al., 1973; 1974; Chan et al., 1980; Lee et al., 1980; Dobbs and James, 1987).

Pharmacokinetic parameters have been evaluated according to a two-compartment open model (Table 1).

After injection of free DXR, the distribution half-life calculated to 0.25 h is of the order of literature values (Benjamin et al., 1977; Reich, 1978; Greene et al., 1983; Robert and Hoerni, 1983; Eksborg et al., 1985). In addition, the elimination half-life (56.80 h) and the volume of distribution (~ 1000 litres) are similar to those calculated by other authors (Benjamin et al., 1973; Oosterbaan et al., 1984; Speth et al., 1987). After a single intravenous injection of free DXR, its plasma concentrations quickly decrease, due to a rapid distribution into tissues. In addition, the

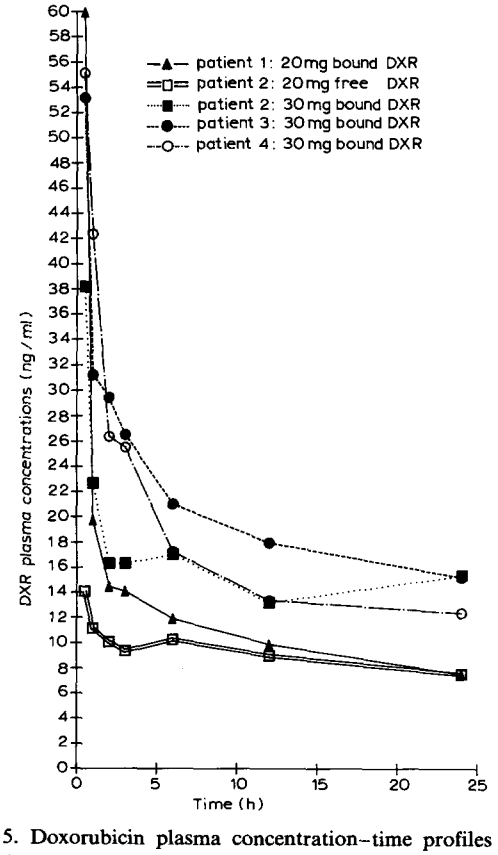

Fig. 5. Doxorubicin plasma concentration–time profiles obtained after a single intravenous injection of free or nanoparticle-bound DXR in hepatoma patients.

TABLE 1

Pharmacokinetic parameters of doxorubicin after a single intravenous administration of free or bound DXR in 4 patients with hepatocellular carcinoma

Parameters (units)	Patient 1 (b)	Patient 2 (a)	Patient 2 (c)	Patient 3 (c)	Patient 4 (c)
Injected dose (D) (mg/kg)	0.385	0.308	0.462	0.395	0.566
A (ng/ml)	393.72	15.26	81.19	174.87	54.72
$T_{1/2}[\alpha]$ (h)	0.16	0.25	0.26	0.18	0.79
B (ng/ml)	15.07	10.30	17.41	28.79	19.80
$T_{1/2}[\beta]$ (h)	22.00	56.80	35.91	21.52	30.40
K_{12} (h^{-1})	3.46	1.62	2.13	3.14	0.57
K_{21} (h^{-1})	0.19	0.12	0.49	0.58	0.25
K_{el} (h^{-1})	0.72	0.03	0.11	0.22	0.08
V (l/kg)	0.94	12.00	4.68	1.9	7.6
AUC_{24h} (μg/l/h)	275.09	216.31	372.37	479.61	408.93
AUC_{24h}/Dose ($\times 10^{-3}$) (l/h)	13.75	10.82	12.41	15.99	13.63
Cl_{24h} (D/AUC_{24h}) (l/h/kg)	1.40	1.42	1.24	0.82	1.38

The intravenously injected doses correspond to: (a) 20 mg of free DXR; (b) 20 mg of bound DXR; (c) 30 mg of bound DXR.

high volume of distribution observed also serves to point out the extensive DXR uptake by tissues.

After administration of bound DXR a prolonged elevation in DXR plasma levels is observed as compared to injected free DXR (Fig. 5). Similar results were previously obtained when comparing the pharmacokinetics of free and nanoparticle-bound DXR in rabbits (Rolland, 1988). After intravenous injection of free or bound DXR, some pharmacokinetic parameters appear different (Table 1). Thus, the elimination half-life ($T_{1/2}[\beta]$) and the volume of distribution (V_c) are reduced after administration of DXR-loaded nanoparticles. The areas under the curves after 24 h, corrected for the injected doses, are higher for the injected nanoparticle-bound DXR than for the free DXR (Table 1). The total clearances (Cl 24 h) are therefore reduced in the case of bound DXR compared to free DXR, these results being similar to those observed previously for the pharmacokinetic study in rabbits (Rolland, 1988).

Since the number of patients receiving DXR-loaded nanoparticles is actually limited, no statistical conclusions can be obtained. Nevertheless, the reduction of the elimination half-life and especially of the volume of distribution and of the total clearance, observed after intravenous injection of doxorubicin-loaded nanospheres in hepatoma patients, leads to the hypothesis of a modification of DXR distribution. Since polymethacrylic nanoparticles have been proved to be naturally removed from the bloodstream by the MPS, mainly by the liver (Rolland et al., 1989), nanoparticle-bound DXR might be rapidly and efficiently taken up by this organ, probably by the Kupffer cells. As DXR is essentially metabolised in the liver (Riggs et al., 1977; Reich, 1978; Loveless et al., 1978), its specific delivery to this organ could result in modifications of its metabolism profile. Indeed in rabbits (Rolland, 1988), the passive targeting of DXR to the liver modified its hepatic concentrations and kinetics of metabolism, the nanospheres behaving as an intrahepatic slow-release system.

The present pharmacokinetic results seem to validate the hypothesis of specific hepatic delivery of nanoparticle-bound DXR. This was already proposed in the corresponding preliminary clinical trials in patients with hepatocellular carcinoma, where a reduction of the toxic side-effects attributed to DXR (nausea, vomitting, cardiomyopathy, medullar depression) and an apparent improved therapeutic activity were observed after intravenous injection of doxorubicin-loaded nanospheres (Rolland et al., 1988).

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