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Separation and determination of liposomal and non-liposomal daunorubicin from the plasma of patients treated with Daunoxome

Ricardo Bellott, Paul Pouna, Jacques Robert*

Institut Bergonié and Université Victor Segalen Bordeaux 2, 180 Rue de Saint-Genès, 33076 Bordeaux Cedex, France

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Abstract

Several liposomal formulations of anthracyclines have been developed recently and are currently used in the clinical setting. We describe a technique of separation and quantification of the liposomal and non-liposomal forms of daunorubicin in the plasma of patients treated with DaunoXome, a liposomal formulation of daunorubicin. The method we propose is based upon the property of liposomes to cross reversed-phase C_{18} silicagel cartridges without being retained, while non-liposomal drug is retained on the stationary phase and is eluted with methanol. Extraction of liposomal and non-liposomal daunorubicin from plasma, therefore, is performed in two steps. This technique is rapid, can be automated in order to handle large series of samples, and the plasma can be frozen after sampling by addition of glycerol. The recovery of liposomal daunorubicin as well as the precision, linearity and accuracy of the technique appear satisfactory for pharmacokinetic purposes.

Keywords: Daunorubicin; Daunoxome

1. Introduction

Liposomal formulations were proposed several years ago as a means for decreasing the toxicity of anthracyclines, especially their cardiotoxicity [1,2]. The rationale for the development of such formulations lies in the preferential uptake of liposomes in tumors as compared with normal tissues [3]. This allows the generation of drug concentrations in tumors much higher than after conventional drug administration, while drug concentrations in normal tissues, especially the heart, remain lower than after conventional drug administration [4]. In addition, it has been shown by several authors that anthracycline liposomal formulations could overcome the multidrug resistance phenotype exhibited by numerous types of cancer cells [5,6], although this observation was not always made [7].

Two different formulations of liposomal anthracyclines have been approved for the treatment of acquired immune deficiency syndrome (AIDS)-related Kaposi's sarcoma: DaunoXome, a liposomal formulation of daunorubicin [8], and Doxil or Caelyx, a pegylated liposomal formulation of doxorubicin [9]. Both of them have shown an important activity in this pathology and are presently under investigation in other solid tumors and hematological malignancies. Both of them have also shown reduced

*Corresponding author. Tel.: +33-5-5633-3327; fax: +33-5-5633-3389.

E-mail address: roben@bergonie.org (J. Robert).

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cardiac toxicity in preclinical models [10,11] as in the clinical setting [12,13]. The pharmacokinetics of liposomal anthracyclines have been studied by several authors during the phase I and phase II clinical trials of these drugs [13–17]. However, only the pharmacokinetics of total drug have been studied up to now, without any attempt to separate the circulating liposomal drug from the non-liposomal drug also present in plasma. The total drug concentrations observed in plasma range between 5 and 50 000 ng/ml after a therapeutic injection [13–15]. It could be of interest to evaluate separately the amounts of the two species, in order to understand the disposition of liposomal formulations and to know whether non-liposomal drug originating from the liposomes can reach therapeutic levels in the blood and contribute, at least in part, the efficacy and toxicity of the liposomal formulation.

Several methods have been proposed for the separation of liposomal and non-liposomal forms of anthracyclines [18,19], but they have not yet been validated for pharmacokinetic purposes. Therefore, we have developed a method for the separation and determination of liposomal drug from non-liposomal drug in the plasma of patients treated with DaunoXome, allowing the study of the pharmacokinetics of each species in these patients.

2. Experimental

2.1. Chemicals and reagents

DaunoXome was obtained from NeXstar Pharmaceuticals (presently Gilead Pharmaceuticals, Boulder, CO, USA) in ready-for-use vials containing 50 mg of liposomal daunorubicin at a concentration of 2 mg/ml. These liposomes are made of distearoylphosphatidylcholine and cholesterol (molar ratio 2:1) and details of their preparation can be obtained from the manufacturer. DaunoXome was diluted in 5% glucose solution, as recommended for clinical use, to reach final concentrations of daunorubicin of 1 and 10 μg/ml.

Daunorubicin's usual clinical formulation (Cerubidine) was obtained from Rhône-Poulenc (presently Aventis Pharma, Vitry-sur-Seine, France) as well as daunorubicin and daunorubicinol pure authentic standards. Doxorubicin authentic standards were obtained from Farmitalia (presently Pharmacia & Upjohn, Milan, Italy).

All reagents and solvents were of analytical grade. Triton X-100 and glycerol were purchased from Sigma (Saint-Quentin-Fallavier, France), while methanol, acetonitrile, 25% ammonia, and formic acid were purchased from Merck (Darmstadt, Germany). Phosphate-buffered saline (PBS) was obtained from Biomérieux (Marcy l'Étoile, France) as a ready-to-dissolve powder. Water was of Milli-Q grade (Millipore, Saint-Quentin-en-Yvelines, France). Sep-Pak cartridges containing 360 mg of reversed-phase C_{18} silicagel were obtained from Waters (Saint-Quentin-en-Yvelines, France). Before use, they were conditioned by passing 3 ml methanol then 6 ml PBS through them. All liquids were passed through the Sep-Pak cartridges under manual pressure.

The mobile phase for the chromatography was a mixture of [0.1% (v/v) ammonia–formic acid buffer, pH 4.0]–acetonitrile (68:32, v/v). It was filtered and degassed by filtration under vacuum before use.

2.2. Biological materials

Fresh plasma and fresh blood from healthy blood donors were obtained from the Établissement de Transfusion Sanguine de Bordeaux (France), checked for absence of known viral contamination and virus-attenuated by heating. Plasma was kept frozen until used, then was thawed and distributed as 1-ml samples in polystyrene tubes. Blood was kept at 4°C until use within the day of taking.

Plasma samples were obtained from patients treated with DaunoXome during a phase II study in breast cancer conducted by Dr. I. Vergote in Leuven (Belgium) and reported elsewhere [20]. Patients received a 2-h infusion of DaunoXome (100 mg/m^2) and samples were obtained before infusion, at the end of infusion, and 4, 7, 24, 48 and 72 h after the onset of infusion. They were processed extemporaneously as explained below. The pharmacokinetics were studied using a non-compartmental model; areas under the curves (AUCs) were estimated using the trapezoidal rule, terminal half-lives by linear

regression, and the total plasma clearance and apparent volume of distribution with the usual combinations of these parameters [21]. When the concentration in plasma exceeded the upper limit of calibration (20 000 ng/ml), the plasma sample was diluted in the pre-treatment plasma sample of the patient.

2.3. Principles of the technique of separation of liposomal and non-liposomal daunorubicin

The method we propose is based upon the property of liposomes to cross reversed-phase C_{18} silicagel cartridges without being retained [18,19], while non-liposomal drug is retained on the stationary phase and eluted with methanol as described previously [22]. Therefore, extraction of liposomal and non-liposomal daunorubicin from plasma is performed in two steps, as outlined in Fig. 1, all liquids being passed through Sep-Pak cartridges under manual pressure.

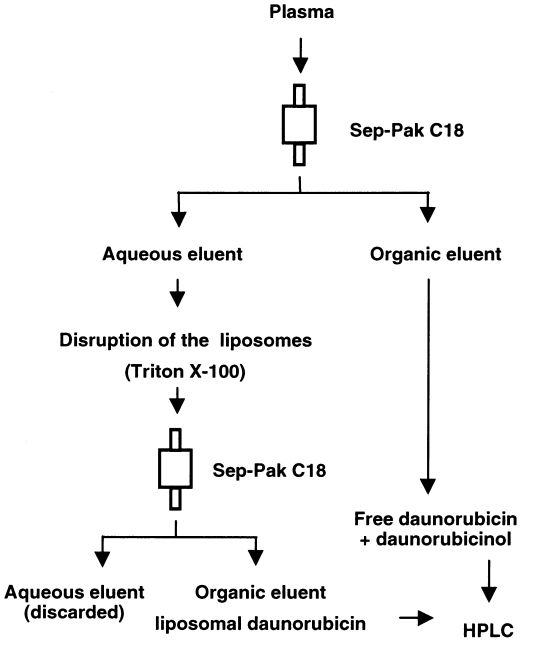

Fig. 1. General scheme for the separate extraction of liposomal and non-liposomal daunorubicin from plasma of patients treated with DaunoXome.

- Plasma is first applied to a cartridge and the liposomes eluted with PBS; non-liposomal drug and eventual metabolites are then eluted with methanol.
- The liposomes in the aqueous eluent are disrupted with a detergent (Triton X-100) and the resulting fluid is applied to a second cartridge; the daunorubicin content of the liposomes is then eluted with methanol.

2.4. Procedure for separation of liposomal and non-liposomal daunorubicin

Plasma samples (1 ml) containing mixtures of non-liposomal and liposomal daunorubicin, as well as a known amount (100 or 1000 ng) of doxorubicin (internal standard) were aspirated in a 5-ml polypropylene syringe and passed through a conditioned Sep-Pak C_{18} cartridge. The eluent, containing liposomal daunorubicin, was collected in a 13-ml polypropylene tube containing 100 μl of 20% (v/v) Triton X-100 in water. The plasma tube was rinsed with 1 ml PBS which was also passed through the cartridge; then 4 ml of PBS was finally passed through the cartridge and all eluents were collected together in the Triton X-100-containing tube. Non-liposomal drugs (daunorubicin, its metabolite daunorubicinol if present, and the internal standard doxorubicin) were then eluted into a 5-ml polystyrene tube with 2×1.5 ml methanol which were evaporated to dryness under a stream of nitrogen.

The liposomal fraction recovered in Triton X-100 was vortex-mixed vigorously, supplemented with a known amount of doxorubicin (internal standard), aspirated in a 5-ml polypropylene syringe and was slowly passed through a new conditioned Sep-Pak C_{18} cartridge. The eluent was discarded, as were the eluents obtained by rinsing the tube and the cartridge with 1-ml fractions of PBS. Methanol (2×1.5 ml) was then passed through the cartridge and the eluate was collected in a 5-ml polystyrene tube, and was evaporated to dryness.

The two dried residues were reconstituted with 200 μl of mobile phase and 10–25 μl was injected onto the high-performance liquid chromatography (HPLC) system.

2.5. Development of a semi-automated method

In order to be able to handle numerous samples within a short period of time, we have developed a semi-automated procedure using a solid-phase extraction device (Aspec XL; Gilson, Villiers-le-Bel, France). In this procedure, the usual Sep-Pak C_{18} cartridges have been replaced by ready-to-use polypropylene syringes containing 500 mg of reversed-phase C_{18} silicagel (Sep-Pak C_{18} Vac; Waters) and the solvents were poured directly onto the syringe by the pumping system through a tight cap. All liquids were passed through Sep-Pak Vac syringes under gravity. It was necessary to proceed in two steps, the liposomal eluent being reintroduced in the sample processor at the end of the first extraction step. The robot system program details may be obtained upon request.

2.6. High-performance liquid chromatography

Quantification of daunorubicin originating either from liposomes or from circulating non-liposomal drug was achieved by HPLC using a column of reversed-phase C_{18} silicagel, Nova-Pak Radial-Pak (Waters), 100×5 mm I.D., 4 μm particle size, inserted in a radial compression device (Waters). The mobile phase was delivered by a Spectra System P1000XR pump (ThermoQuest, Les Ulis, France) at a flow-rate of 2 ml/min. Detection was carried out with a laser-induced fluorescence detector (Zeta Technology, Toulouse, France) with excitation and emission wavelengths set at 488 and 560 nm, respectively. Samples were injected via an automatic sampler (Spectra System AS 1000, ThermoQuest) and peaks were recorded and quantified using PC 1000 software (ThermoQuest).

3. Results

3.1. Calibration curves for daunorubicin

Two calibration curves for daunorubicin and one for daunorubicinol were first obtained after spiking known amounts of daunorubicin (10 to 500 and 500 to 20 000 ng) or daunorubicinol (10 to 500 ng) into 1-ml samples of plasma containing a fixed quantity of doxorubicin (100 ng and 1000 ng, respectively, for the daunorubicin calibration curves and 100 ng for the daunorubicinol calibration curve). Extraction was performed according to the classical technique [22]. The characteristics of the calibration curves are presented in Table 1. All characteristics of extraction (reproducibility, recovery) were identical to those already established during the development of the solid-phase extraction technique for anthracyclines and metabolites [22] and widely used afterwards. The limit of detection of daunorubicin and daunorubicinol was 0.1 ng, and the lower limit of quantification was 0.5 ng/ml.

3.2. Extraction of DaunoXome from plasma

We spiked known amounts of DaunoXome (10 to 20 000 ng) in 1-ml samples of plasma containing a known quantity of doxorubicin (100 ng for the tubes containing 10 to 500 ng of DaunoXome, 1000 ng for the tubes containing 500 to 20 000 ng of DaunoXome). Extraction was performed according to the two-step method described in the Experimental section. Another known amount of doxorubicin (100 ng) was added to the liposomal fraction between the two steps to enable quantification. Fig. 2 presents several typical chromatograms of a blank plasma extract, standards spiked in blank plasma and

Table 1
Characteristics of the calibration curves for daunorubicin and daunorubicinol

	Number of points	Slope	Intercept	Coefficient of correlation
Daunorubicin 10–500 ng	7	0.9789	−1.41	0.9996
Daunorubicin 500–20 000 ng	7	1.159	−80.9	0.9999
Daunorubicinol 10–400 ng	7	0.935	8.26	0.9904

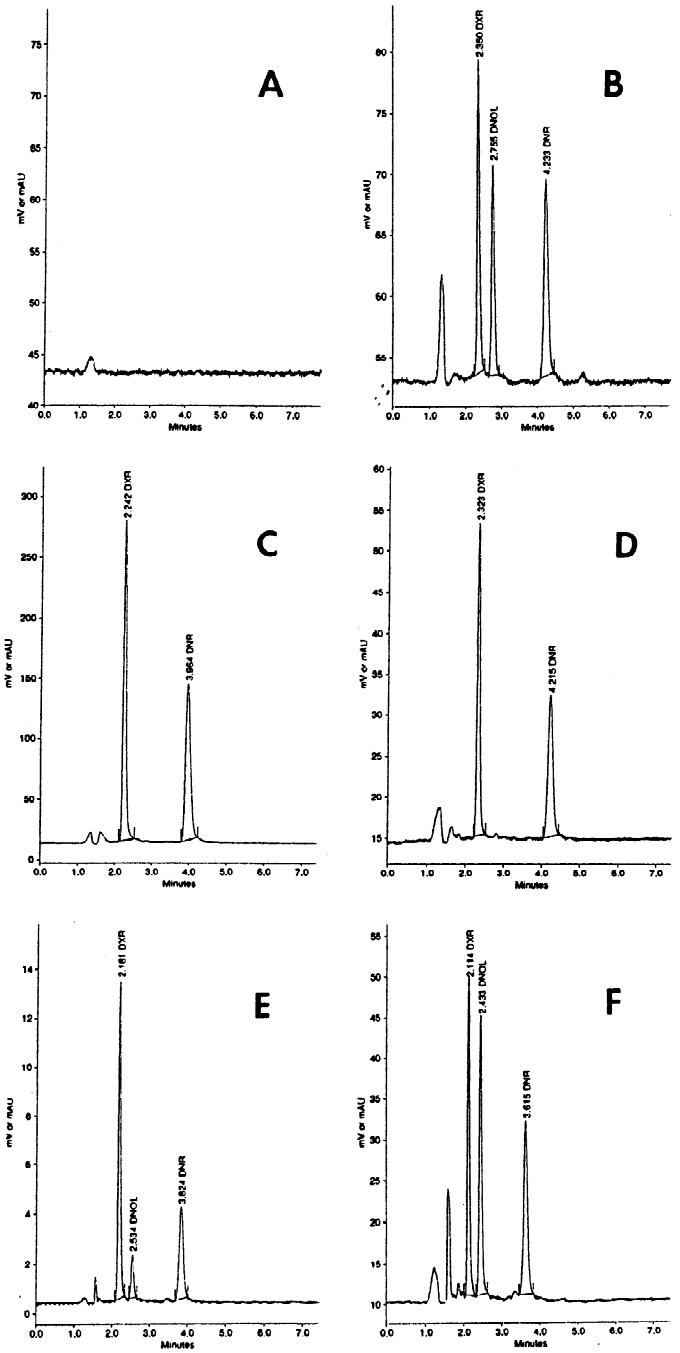

Fig. 2. Typical chromatograms of several extracts. In all cases, the dry residue was reconstituted with 200 μl mobile phase and 20 μl was injected. DXR: doxorubicin (internal standard); DNR: daunorubicin; DNOL: daunorubicinol. (A) Blank plasma extract (1 ml). (B) Standard mixture of doxorubicin, daunorubicin and daunorubicinol, 10 ng each, extracted from 1 ml blank plasma. (C) Extract of 1000 ng of DaunoXome spiked in 1 ml blank plasma: liposomal fraction, internal standard 1000 ng. (D) Same extract as in (C): non-liposomal fraction, internal standard 100 ng. (E) Extract of a plasma sample (1 ml) taken 24 h after administration of 100 mg/m^2 DaunoXome to a patient: liposomal fraction, internal standard: 100 ng. (F) Same extract as in (E): non-liposomal fraction, internal standard: 100 ng.

extracts of a plasma sample originating from a patient treated with DaunoXome.

From three series of experiments at 12 different concentrations, we observed that 92.5±1.6% (mean±SD) of total daunorubicin was recovered in the liposomal fraction and 7.41±1.56% in the non-liposomal fraction (Fig. 3). The sum of daunorubicin levels present in the two fractions represented 80.8±8.9% of the amount of DaunoXome added in plasma, with no significant change over the whole range of concentrations.

This result raises the problem of the origin of the non-liposomal daunorubicin observed after adding only DaunoXome to the plasma samples. Either a definite amount of non-liposomal daunorubicin exists in the DaunoXome pharmaceutical preparation, or the technique of extraction damages the liposomes and cannot be used accurately.

To address this question, we have verified liposome integrity during the extraction process as follows. After the first run of DaunoXome-containing plasma through a Sep-Pak cartridge, the liposomal fraction was collected in the absence of Triton X-100 (i.e., the liposomes were not disrupted) but processed exactly like a new fresh plasma sample by two-step extraction according to the technique described in the Experimental section. Under these conditions, the amount of non-liposomal daunorubicin found in the corresponding eluent represented 0.58±0.45% of the total amount of daunorubicin recovered. This shows that most of the non-liposomal daunorubicin found in plasma spiked with DaunoXome originated from the DaunoXome preparation itself and that only a very small amount originated from mechanical disruption of the liposomes in the cartridge.

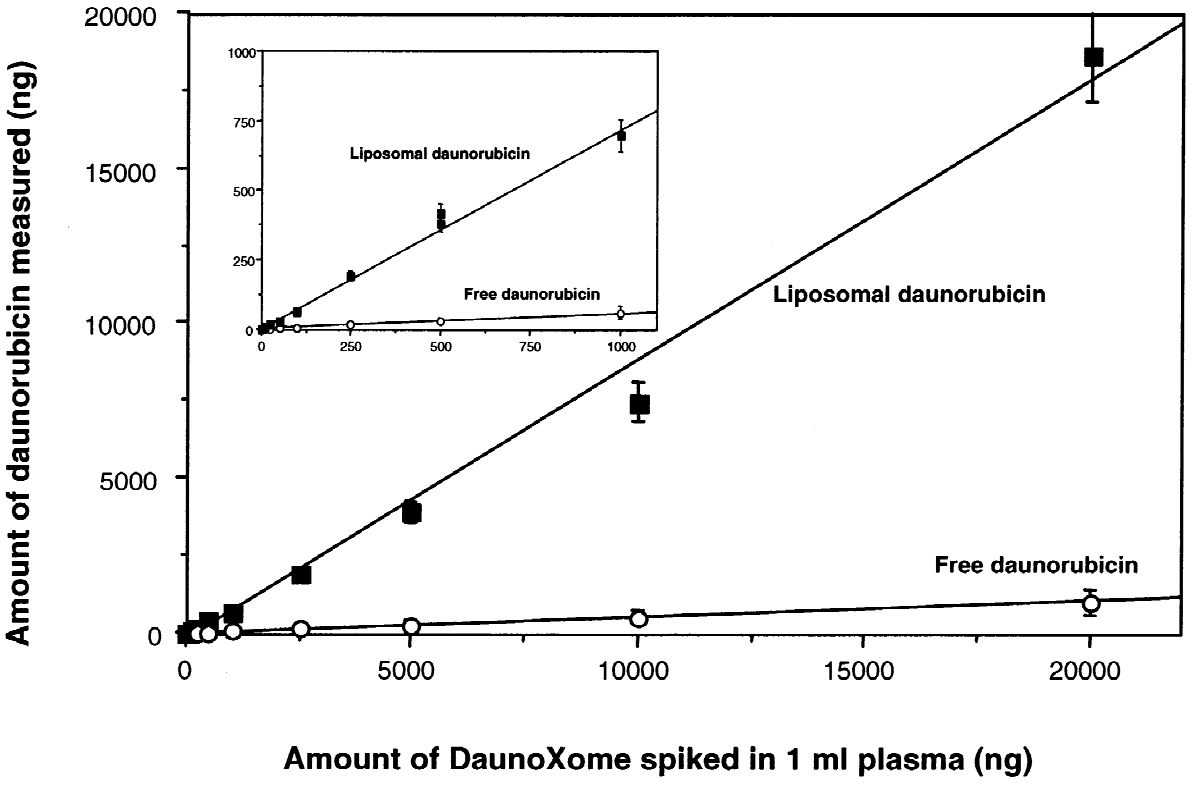

Fig. 3. Recoveries of liposomal (■) and non-liposomal (○) daunorubicin from plasma spiked with different amounts of DaunoXome in the range 10–20 000 ng. Values are means±SD of three determinations.

3.3. Extraction of mixtures of DaunoXome and non-liposomal daunorubicin from plasma

In these experiments, we spiked known amounts of DaunoXome (50 to 5000 ng) and Cerubidine (50 to 5000 ng) into 1-ml samples of plasma containing a known quantity of doxorubicin (100 ng for the tubes containing 50 to 500 ng of DaunoXome and of daunorubicin, 1000 ng for the tubes containing 500 to 5000 ng of DaunoXome and of daunorubicin). Extraction was performed according to the two-step method described in the Experimental section. Another known amount of doxorubicin (100 ng or 1000 ng as in the step before) was added to the liposomal fraction between the two steps for quantification.

From three series of experiments at eight different concentrations, the amount of daunorubicin recovered in the liposomal fraction represented 83.0±7.9% of the amount of DaunoXome added to plasma, while the amount of daunorubicin recovered in the non-liposomal fraction represented 106±8% of the amount of Cerubidine added to plasma (Fig. 4). These experiments confirm that it is possible to accurately evaluate the proportions of liposomal and non-liposomal daunorubicin when both forms are present in plasma, taking into account the fact that a definite proportion of non-liposomal drug is present in the DaunoXome formulation.

3.4. Precision and accuracy of the technique

The within-day and between-day precision and accuracy were estimated for the liposomal and non-

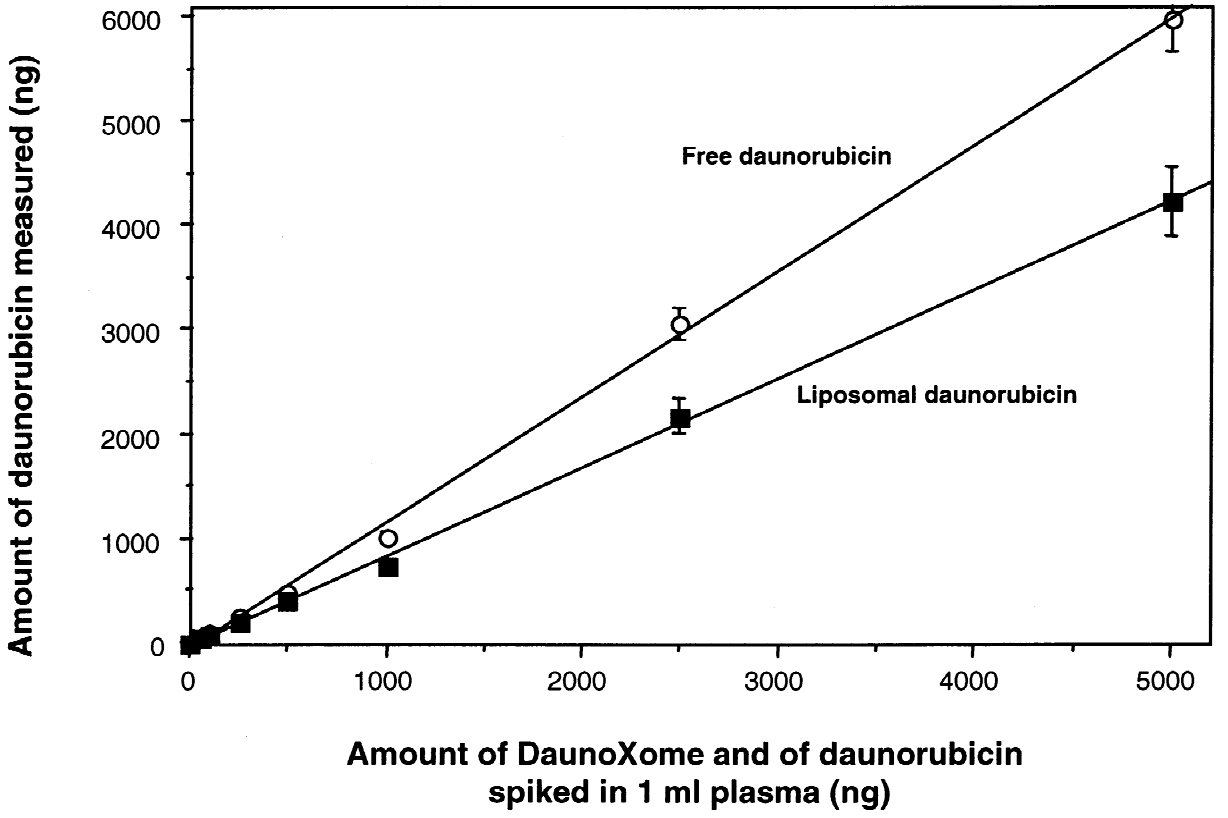

Fig. 4. Recoveries of liposomal (■) and non-liposomal (○) daunorubicin from plasma spiked with equimolar amounts of DaunoXome and free daunorubicin in the range 50–5000 ng. Values are means±SD of three determinations.

liposomal forms of daunorubicin after spiking four different amounts (10 to 10 000 ng) of DaunoXome in five series of 1-ml plasma samples as described above. We present in Table 2 the within-day and between-day precision of the technique for both liposomal and non-liposomal forms. The accuracy was determined also under the same conditions after summing the amounts of both forms and comparing with the quantity of DaunoXome spiked. Both parameters appear satisfactory for analytical purposes. The precision and accuracy for daunorubicinol determination had been validated long ago with this technique (data not shown).

3.5. Plasma freezing

All experiments reported here were made on fresh plasma not having undergone freezing after addition of the drug. In order to know whether it was possible to freeze patients' plasma before analysis, we froze at −20°C 1-ml plasma samples after spiking them with 1000 ng DaunoXome, either in the presence of 200 μl glycerol or not. It should be noted that gentle stirring is necessary before freezing since, otherwise, glycerol and plasma do not mix homogeneously and the cryoprotective effect of glycerol is then lost. When plasma was frozen for 4 weeks without glycerol (12 samples), non-liposomal daunorubicin amounted to 30.7±2.4% of total daunorubicin, while liposomal daunorubicin decreased accordingly. In contrast, when frozen in the presence of glycerol (15 samples), the proportion of non-liposomal daunoinbicin was 11.3±1.5%, a proportion identical to that obtained from fresh plasma (11.7±1.4% in this set of experiments). Very similar results were obtained when spiking 100 or 10 000 ng of DaunoXome in 1-ml plasma samples instead of 1000 ng. We assume, therefore, that it is possible to freeze plasma samples of patients treated with DaunoXome, but only if 200 μl glycerol is added to 1-ml plasma before freezing.

3.6. Comparison of automated and manual procedures

We have evaluated the liposomal and non-liposomal concentrations of daunorubicin in DaunoXome preparations after spiking known amounts of DaunoXome (50 to 20 000 ng) in 1-ml plasma samples and extracting them with the manual and automated procedures. The proportions of the non-liposomal form as well as the extraction recoveries were similar using the two methodologies (data not shown).

3.7. Stability of DaunoXome in whole blood

In order to determine whether it was necessary to centrifuge blood rapidly after sampling in order to keep the liposomes as intact as possible, we directly spiked a known amount of DaunoXome (10 000 ng) into 2.5-ml samples of whole blood collected on EDTA, and centrifuged immediately or at various times after spiking (1, 2, 4, 6 and 24 h). Under these conditions, there was no difference in the recovery of total daunorubicin in plasma over time (77.7±3.8%) and no difference in the proportion of the non-liposomal form in plasma (3.44±0.22%). This indicates good stability of liposomes in blood and shows that no special care for rapid centrifugation after blood sampling is required. It should be noted that the proportion of non-liposomal daunorubicin was consistently and significantly lower after spiking DaunoXome in whole blood than in plasma. This can

Table 2
Precision and accuracy of the determination of free and encapsulated daunorubicin using the technique proposed

Concentration (ng/ml)	Precision (% RSD of five determinations)				Accuracy (% means of five determinations)	
	Free drug		Liposomal drug		Within-day	Between-day
	Within-day	Between-day	Within-day	Between-day		
10	10.3	17.3	3.9	9.5	130	118
100	4.6	7.7	7.7	4.5	97.2	91.5
1000	3.1	8.6	5.5	7.9	100	98.7
10 000	9.1	15.8	7.7	4.8	96.8	110

be attributed to a rapid adsorption or uptake of non-liposomal daunorubicin in blood cells.

3.8. Studies on patients' plasma

Preliminary pharmacokinetic studies were performed with the plasma samples obtained from six patients suffering from breast cancer and treated with a dose of DaunoXome of 100 mg/m^2 over 2 h. Fig. 5 presents the time course of liposomal daunorubicin, non-liposomal daunorubicin and daunorubicinol in plasma. Different kinetic aspects were obtained for liposomal and non-liposomal daunorubicin. Liposomal daunorubicin was eliminated following a one-exponential decay while non-liposomal daunorubicin, whose concentrations barely reached 1% of those of liposomal daunorubicin at the end of the infusion, followed a two-exponential decay, its concentration being in the same order of magnitude as that of liposomal daunorubicin 72 h after the onset of infusion. The $AUC_{0-\infty}$ of non-liposomal daunorubicin represented 1.33% and that of daunorubicinol 1.75% of the total $AUC_{0-\infty}$ of the total drugs. Elimination half-lives of liposomal daunorubicin, non-liposomal daunorubicin and daunorubicinol were estimated to be 5.9, 14.7 and 21.8 h, respectively. The total plasma clearance of liposomal daunorubicin was 0.4 l/h/m^2 and its total volume of distribution at steady-state was about 4 l/m^2. The complete clinical and pharmacokinetic study will be published elsewhere.

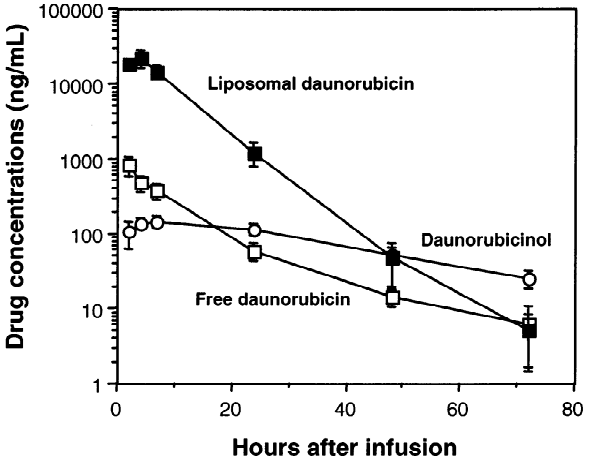

Fig. 5. Time–course of the fluorescent species found in plasma after administration of DaunoXome: (■) liposomal daunorubicin; (□) non-liposomal daunorubicin; (○) daunorubicinol. Values are means±SD of the plasma levels measured in six patients treated at the dose of 100 mg/m^2.

4. Discussion

Liposomal formulations of anthracyclines have been approved recently for the treatment of AIDS-related Kaposi's sarcoma and are under evaluation for the treatment of other malignancies. A complete understanding of the disposition of these formulations in humans requires a comprehensive plasma pharmacokinetics analysis. As pointed out by Druckman et al. [18], several factors contribute to the complexity of the pharmacokinetics of liposomal formulations: (1) circulating drug is present under three forms: liposome-associated, protein-bound, and free drug; (2) plasma clearance of liposomal drug occurs as a result of three processes with different elimination rates: (i) tissue uptake of drug-containing liposomes; (ii) release of drug from liposomes, occurring either in plasma (leakage) or after liposome uptake in tissues (especially the reticulo–endothelial system); (iii) clearance of non-liposomal drug. The elimination rate of this last process is known from pharmacokinetic studies of the non-liposomal drug, but the rates of elimination of the two other processes are unknown.

In order to obtain a meaningful pharmacokinetic profile of liposomal and non-liposomal forms of daunorubicin when administered as DaunoXome, we have implemented a technique able to separate these forms and to accurately determine their daunorubicin content. This technique is rapid, can be automated in order to handle large series of samples, and the plasma can be frozen between sampling and analysis. The recovery of liposomal daunorubicin as well as the precision, linearity and accuracy of the technique appear satisfactory for pharmacokinetic purposes, keeping in mind that these factors were already optimized for non-liposomal anthracyclines [22], using the solid–liquid extraction technique for anthracyclines now widely used.

Different procedures have been proposed for the separation of liposomal and non-liposomal drugs in plasma including differential centrifugation, size-ex-

clusion chromatography, ultrafiltration, and ion-exchange chromatography [18], but no technique has presently been validated for pharmacokinetic purposes. The procedure we propose uses rather a process of partition or affinity chromatography, since it is based upon the interaction of free anthracyclines with the long chains (C_{18}) grafted onto the silicagel support, which is only disrupted by strong solvents such as methanol. This technique is more rapid and reproducible than others, especially because it uses cartridges specifically designed for solid–liquid extractions of drugs from plasma, which allows reproducible pharmacokinetic analyses of numerous patient samples. It must be noted, however, that this technique cannot separate protein-bound drug from actually free drug: both forms are included in the non-liposomal fraction. This is the same for all the techniques developed for measuring anthracycline concentrations in plasma or other biological fluids, with either solid–liquid or liquid–liquid extraction techniques [23]. The development of this technique allowed us to demonstrate that a small but significant proportion of daunorubicin (around 10%) in DaunoXome was not associated with liposomes but could be recovered in the non-liposomal fraction. Indeed, the percentage of non-liposomal daunorubicin generated by artificial disruption of the liposomes during the extraction process remained below 1%.

In the preliminary pharmacokinetic study presented here, we were able to show that the time–course profiles of liposomal and non-liposomal drug were quite different. The decay of liposomal daunorubicin appears roughly similar to that of total daunorubicin observed after DaunoXome administration [13–15], characterized by a monoexponential curve with a rapid elimination half-life and a small volume of distribution. This is not surprising since we found that the area under the curve of the liposomal form represented 97% of the sum of the AUCs of the two forms. In contrast, non-liposomal daunorubicin follows a decay similar to that observed after administration of conventional daunorubicin [24,25] with a prolonged elimination half-life. This is in agreement with the presence of a small amount of free drug in the liposomal formulation, but we cannot exclude the contribution of a release of free drug from the liposomal formulation, either directly in plasma or after disruption of the liposomes in tumor tissue or in reticulo–endothelial cells. This question is presently under investigation in our laboratory.

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