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Separation of liposome-associated doxorubicin from non-liposome-associated doxorubicin in human plasma: implications for pharmacokinetic studies *

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To characterize the pharmacokinetics of liposome-associated drugs, the fraction of drug circulating in liposome-associated form and the absolute plasma drug levels must be determined. In this report, we describe our methodological approach to quantitate plasma liposome-associated doxorubicin separately from protein-bound and free doxorubicin. The method is based on the affinity of a cation-exchange resin for doxorubicin and the repulsion by the same resin of negatively-charged liposomes. The methodology is technically simple and reproducible, and lends itself to the analysis of multiple plasma samples as required in pharmacokinetic studies. The validity of this approach was confirmed by separation of liposome-associated from non-liposome-associated drug using gel exclusion chromatography.

Liposomal drug delivery systems have recently entered clinical trials with the aim of improving the therapeutic index of various chemotherapeutic agents [1–5]. A complete evaluation of these trials will require a comprehensive plasma pharmacokinetic analysis. There are several factors contributing to the complexity of the pharmacokinetics of drugs delivered by liposomes after i.v. administration:

(1) Circulating drug is present in three forms: liposome-associated, protein-bound, and free drug.

(2) Plasma clearance occurs as a result of three processes with different elimination rates: (i) tissue uptake of liposomes carrying the drug, (ii) leakage of drug from liposomes, and (iii) clearance of free drug. Although the rate constant of the latter is known from previous pharmacokinetic analysis of the free drug, we still need to determine the rates of the other two processes to obtain a meaningful pharmacokinetic picture.

A pharmacokinetic study limited to the analysis of total drug concentrations in plasma is not informative enough and may even be misleading, since the pharmacological effects are mainly related to the level of free drug in plasma. Also, the tissue distribution of small molecular weight drugs is markedly different when the drug is entrapped in liposomes [6]. Liposomes are taken up preferentially by liver and spleen, with minimal uptake by tissues with continuous, nonfenestrated capillaries such as muscle and nervous tissue [7]. Therefore, the relative plasma levels of liposome-associated and free drug may have a predictive value on the pattern of tissue distribution achieved. For these reasons, it is crucial to develop proper methodology to determine the levels of liposome-associated drug and free drug in parallel to total plasma drug measurements. The most direct approach is to separate the liposome-associated drug from the non-liposome-associated drug. This work describes the development of such a method for a liposome-doxorubicin formulation and demonstrates its use in patients.

The liposomes used in this study [8] are composed of egg phosphatidylcholine (PC), egg PC-derived phosphatidylglycerol (PG), cholesterol, d-alpha-tocopherol succinate, and doxorubicin-HCl (DXR). The molar ratio of the various lipid components (PC/PG/cholesterol/tocopherol succinate) is 7:3:4:0.2, respectively. The final DXR/phospholipid molar ratio is approximately 5:100, after removal of the nonencapsulated drug. The liposomes are prepared by thin lipid film hydration and

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extrusion through 0.2 μm-pore polycarbonate membranes. Their average size is 250 nm.

Two approaches were applied to separate between liposome-associated and non-liposome-associated (protein-bound and free) DXR.

(i) Separation by size. Except for small unilamellar vesicles, liposomes can be separated from most high molecular weight plasma components by gel exclusion chromatography [9].

(ii) Separation by charge. The presence of an acidic phospholipid (PG) in molar excess over DXR (6:1) confers to the liposomes used here a net negative charge. In contrast, free DXR is positively charged below its pK (pH = 8.25) [10]. Therefore, liposomes carrying drug and free drug have opposite charges and can be separated by ion exchange chromatography. However, a crucial unknown in this approach is the fate of protein-bound drug and the effect of plasma components on this separation.

Human plasma from patients receiving i.v. infusions of liposome-associated DXR or normal volunteers was separated from blood cells by centrifugation at 2000 rpm and 4°C. To eliminate any artifacts, both gel exclusion chromatography and ion-exchange chromatography were performed immediately after plasma separation using non-frozen plasma. In vitro samples were prepared by adding the drug or metabolite to be tested and/or liposome-associated drug either to plasma, or isopropanol. Gel chromatography was performed on columns of Bio-Gel A-15 (Bio-Rad, Emeryville, CA). For ion exchange chromatography, a cation-exchange resin, Dowex 50W × 4, 200-400 mesh from Sigma (St. Louis, MO) was used. It has been previously shown that Dowex removes free DXR in buffer solution by a combination of electrostatic and hydrophobic interactions [11].

To test the ability of Dowex to remove free and protein-bound DXR from plasma, we added 0.3 g Dowex to the top of a Bio-Gel column. Fig. 1 clearly shows that more than 95% of the drug is removed from plasma samples preincubated with free DXR. This indicates that protein-bound and free DXR are removed by Dowex. DXR in plasma is known to be approx. 80% protein-bound [12] with most of the drug being associated to proteins of low molecular weight such as albumin [13]. As a result of this, we were not able to achieve a satisfactory resolution between the protein-bound and free drug peaks using Bio-Gel.

To examine whether the Dowex resin removes plasma proteins, we measured the plasma protein content before and after Dowex treatment either by the Lowry procedure [14] or by standard medical analysis (Technicon SMAC-1 analyzer). There was no significant difference in the amount of protein recovered, indicating that nonspecific binding of proteins to the resin cannot account for the removal of protein-bound DXR from

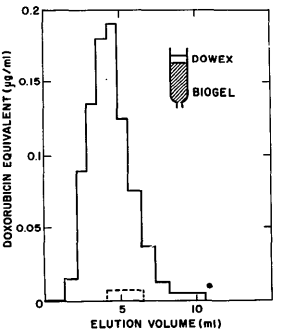

Fig. 1. Chromatography of plasma preincubated with free DXR with and without Dowex treatment. Free DXR (doxorubicin-HCl, Farmitalia-Carlo Erba, Milan, Italy) was added to fresh human plasma at a concentration of 1 μg/ml and incubated for 10 min at 37°C. Protein plasma concentration was in the range of 60 to 80 mg/ml as determined by the Lowry procedure [14] or by standard medical analysis (Technicon SMAC-1 analyzer). A volume of 0.5 ml of plasma was eluted with saline using a 15×0.6 cm Bio-Gel A-15 (Bio-Rad, Emeryville, CA) column. Dowex 50W×4, analytical grade, 200–400 mesh, from Dow Chemical Company, was obtained through Sigma (St. Louis, MO). Prior to use, the resin was washed with NaOH (2 M), NaCl (1 M) and isotonic NaCl (0.9%) until the pH becomes neutral. The plasma sample was mixed with 0.3 g Dowex in a 6×1 cm minicolumn and incubated for 10 min at room temperature. The sample was then collected by centrifugation (2000 rpm, 10 min) to ensure complete elution. In some instances, 0.3 g Dowex was added to the top of the Bio-Gel column prior to plasma fractionation, instead of the use of a separate minicolumn. DXR was extracted from the eluted fractions with acidified ethanol (0.3 M HCl in 50% ethanol) and quantitated by fluorescence intensity as previously described [18]. Solid line, gel fractionation without prior Dowex treatment; broken line, gel fractionation after Dowex treatment.

plasma. It is likely that DXR is removed by displacement from protein-binding sites into the active binding sites of the resin. It should also be pointed out that between 5 to 10% of the plasma water is lost when plasma samples are treated with the Dowex resin.

Fig. 2 shows the fractionation of an in vitro mixture consisting of liposome-associated, protein-bound, and free DXR in plasma using Bio-Gel exclusion chromatography (solid line). The first peak containing the liposomal drug is eluted with the void volume of the column (V_o = 2.85 ml). The following fractions (V_e = 3.85 ml) consist of free and mostly protein-bound drug. For comparison, it should be noted that the V_e of free DXR in saline is 4.45 ml. When the plasma sample is pretreated with Dowex (broken line), Bio-Gel chromatography shows only the liposome-associated drug peak.

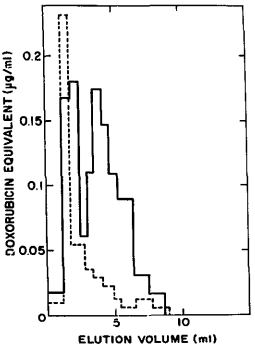

Fig. 2. Chromatography of plasma preincubated with liposome-associated and free DXR with and without Dowex treatment. Liposome-associated and free DXR were added to fresh human plasma, each at a concentration of 1 μg/ml and incubated for 10 min at 37°C. The phospholipid content applied to the column was about 35 nmol per 1 μg DXR. For liposome lipid composition, see text. Total volume of plasma loaded on the column was 0.5 ml. Other experimental details were as in the legend to Fig. 1. Solid line, gel fractionation without prior Dowex treatment; broken line, gel fractionation after Dowex treatment.

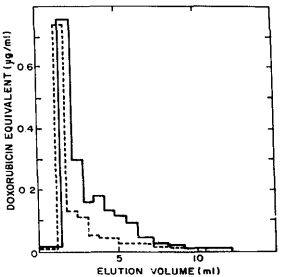

Fig. 3. Chromatography of plasma from a patient at the end of an infusion of liposome-associated DXR with and without Dowex treatment. The patient received a dose of 85 mg/m^2 body surface of liposome-associated DXR during a one-hour long i.v. infusion. Blood samples were obtained from the arm vein contralateral to the infusion side. Plasma was separated immediately by centrifugation (2000 rpm at 4°C). A volume of 0.5 ml plasma was fractionated on a Bio-Gel column before (solid line) and after (broken line) Dowex treatment.

Fig. 3 presents the chromatography of a patient plasma sample taken immediately at the end of a one hour-long i.v. infusion of liposome-associated DXR. Most of the drug (> 90%) is not retained by Dowex, thus indicating that liposome-associated drug accounts for most of the drug found in plasma.

To determine whether Dowex treatment of plasma can be used without gel chromatography, it is essential to examine the fate of drug metabolites. Thus, high pressure liquid chromatography (HPLC) analysis combined with Dowex treatment was used to determine the ability of Dowex to remove free daunorubicin, DXR and some of its metabolites (doxorubicinol, doxorubicin aglycone, doxorubicinol aglycone, 7-deoxydoxorubicin aglycone). Due to the insolubility of the nonpolar aglycones in plasma and water phases, the Dowex treatment was done in the presence of isopropanol or plasma depending on the solubility of each compound. While Daunorubicin, DXR and doxorubicinol are removed with an efficiency close to 100% either in plasma or isopropanol, the three nonpolar aglycones are only partially removed by the resin (Table I). This incomplete removal of DXR metabolites is probably due to the lack of the daunosamine moiety in the aglycones, resulting in a reduced electrostatic interaction with Dowex. Therefore, Dowex treatment followed by fluorimetric quantitation can be misleading since aglycones, either protein-bound or not, are only partially removed resulting in an overestimation of the fraction of liposome-associated DXR. This problem is completely avoided by HPLC analysis of the resin-treated samples before proceeding with fluorimetric quantitation.

Table II provides an example of the results of plasma determinations of DXR following resin treatment and HPLC in a patient receiving liposome-associated DXR. It can be noticed that the levels of DXR decrease rapidly, approximately 30-fold, within the first hour after infusion. The fraction of drug associated to liposomes accounts for most of the circulating drug during

TABLE I

Efficiency of removal of daunorubicin, DXR and metabolites by the Dowex resin

Drug/metabolite [a]	% remaining after Dowex treatment	
	plasma	isopropanol
Doxorubicin	3	3
Doxorubicinol	1	2
Doxorubicin aglycone	n.d. [b]	34
Doxorubicinol aglycone	n.d.	38
7-Deoxydoxorubicin aglycone	n.d.	44
Daunorubicin	3	5

[a] The drugs or metabolites were dissolved in plasma or isopropanol at a concentration of 1 μg/ml and separated by HPLC following the method of Beijnen et al. [16] with modifications.

[b] Not done due to solubility problems.

TABLE II

Plasma levels of liposome-associated DXR in a patient using the Dowex resin method [1]

Time after infusion (min)	Total DXR concn. (μg/ml plasma)	% liposome-associated drug (S.D.)
−25 (mid-infusion)	5.0	89 (10)
0	4.5	100 (10)
15	1.95	100 (10)
60	0.15	67 (7)
240	0.034	38 (5)

[a] Liposome-associated DXR was administered i.v. at a dose of 85 mg/m^2 body surface.

and immediately after infusion. Four hours after the end of the infusion, it represents only one-third of the circulating drug and less than 1/100 of the peak levels of liposome-associated drug. These results stress the importance of obtaining a separate measurement for liposome-associated drug, especially since many of the side-effects of DXR are correlated with high peak levels of free drug [15].

The main disadvantage of the Dowex method is that the level of nonliposome-associated drug (free and protein-bound) has to be directly estimated by substracting the concentration of liposome-associated drug from the total drug concentration. This may cause a significant error, especially when the levels of nonliposome-associated drug are much lower than those of liposome-associated drug. The development of methods to recover the resin-bound drug should obviate this problem. However, our current efforts to recover intact drug from the resin have so far been unsuccessful. We found that DXR elution from the Dowex resin requires pH higher than 11.0. The best recoveries were obtained using ammonium hydroxide in isopropanol. Similar, though less efficient, elution was obtained with ammonium hydroxide in methanol or water. This procedure led to fast degradation of DXR to various products as analyzed by HPLC [16] and by thin-layer chromatography [17]. Therefore, this methodology is as yet not useful for pharmacokinetic evaluation.

In the case of gel exclusion chromatography, metabolites do not interfere with the determination of liposome-associated DXR. This method is, however, impractical for the analysis of a large number of samples as required in pharmacokinetic studies. Other problems of the gel chromatography approach are sample dilution, and lack of resolution between small liposomes (< 100 nm diameter) and low-density lipoproteins [9].

In conclusion, we recommend the Dowex resin treatment followed by HPLC analysis to obtain adequate data for pharmacokinetic analysis of liposome-associated doxorubicin. This methodology should allow for a better insight into the distribution, clearance, and in vivo rate of leakage of liposome-associated DXR in humans, especially if combined with measurement of the plasma levels of liposome constituents or stable liposome markers.

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