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The interaction of cytostatic drugs with adsorbents in aqueous media. The potential implications for liposome preparation

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A new method that involves the use of the cation exchange resin Dowex 50W-X4 to remove non-encapsulated drugs from liposome dispersions was investigated. Cytostatic drugs widely varying in their molecular structure can be removed from aqueous solutions by Dowex 50W-X4. The applicability of the resin to separate free from liposome-bound drugs was illustrated for a number of cytostatic drugs (cisplatin, doxorubicin, vincristine). The technique presented allows for a rapid, efficient and convenient procedure for the free drug removal from liposome dispersions without dilution of the liposomal preparation. Studies with liposome-encapsulated drugs will be facilitated by the use of this method, since it avoids many of the problems introduced by conventional methods as dialysis, gel filtration and centrifugation/washing. To elucidate the interaction mechanism of doxorubicin with Dowex 50W-X4, alternative adsorbents were studied for their doxorubicin binding properties. In the adsorption process of doxorubicin onto Dowex 50W-X4 both electrostatic (ion exchange) and hydrophobic effects play a role. The results indicate that hydrophobic contributions to the interaction are responsible for the high resistance offered by the binding forces against desorption of adsorbed doxorubicin. For other adsorbents the interactions are either mainly of an electrostatic or a hydrophobic nature.

Introduction

Liposomes have aroused considerable interest as drug carriers in cancer chemotherapy over the last decade [1–4]. Classical techniques in liposome technology for the removal of free, non-encapsulated drug from the liposome dispersion show some serious drawbacks. Dialysis is time consuming, gel filtration brings about dilution of the sample, Millipore filtration yields sometimes erratic results, while the centrifugation/washing technique often causes aggregation or fusion of the liposomes, increasing the polydispersity of the product [5–7]. Cation exchange resins have been proposed for free drug removal [7,8]. In particular for cationic drugs in combination with negatively charged liposomes this approach might be interesting. The fields of application of this new method and the exact binding mechanism have not been investigated in detail, yet.

In this paper the applicability of the strongly acidic cation exchange resin Dowex 50W-X4 (Dowex) to remove non-encapsulated drugs from liposome dispersions was investigated for a number of antineoplastic drugs that have been encapsulated in liposomes: cisplatin [9,10], cytara-

bine [11,12], doxorubicin [13,14], methotrexate [15,16] and vincristine [17,18].

To gain more insight into the doxorubicin-Dowex adsorption process, alternative adsorbents were studied for their doxorubicin binding properties. The desorption conditions were established. Besides, attempts were made to elucidate the nature of the interactions between drug and adsorbent (electrostatic or hydrophobic).

Materials and Methods

Materials

Source and quality of adsorbents. Dowex: Dowex 50W-X4, analytical grade, 200–400 mesh, wet density (apparent) 0.80 g/ml, converted to the sodium form (Serva, Heidelberg, F.R.G.). Silica gel: Kieselgel 60 H (Merck, Darmstadt, F.R.G.). Aluminium oxide: basic aluminium oxide 60, the acid form was obtained by HCl titration to pH 4 (Merck, Darmstadt, F.R.G.). Sephadex: SP-Sephadex C-50, sodium form (Pharmacia Fine Chemicals, Uppsala, Sweden). Amberlite: Amberlite XAD-2, 20–50 mesh, wet density 1.02 g/ml (Fluka, Buchs, Switzerland).

Source and quality of the cytostatic drugs. Doxorubicin: doxorubicin-hydrochloride (Lab. Roger Bellon, Neuilly sur Seine, Paris, France). Cisplatin, *cis*-diamminedichloroplatinum(II) (Bristol-Myers, New York, NY, U.S.A.). Cytarabine: Cytosar (Upjohn, Kalamazoo, MI, U.S.A.). Vincristine: Oncovin (Lilly, Indianapolis, IN, U.S.A.). Methotrexate: Emthexate (Pharmachemie, Haarlem, The Netherlands). Tritium-labeled cytarabine, vincristine and methotrexate were supplied by Amersham International, Amersham, U.K. with specific activities of 11.2, 7.7 and 16 Ci/mmol, respectively.

Egg L-alpha-phosphatidylcholine (PC), bovine brain L-alpha-phosphatidylserine (PS) and cholesterol (Chol) of highest obtainable purity were supplied by Sigma Chemicals (St. Louis, MO, U.S.A.). Phospholipid purity was evaluated by thin-layer chromatography (chloroform/methanol/water, 65 : 35 : 5, v/v). It was found that PC consisted for less than 2.5% of lyso-compounds; for PS this percentage was below 5% [21].

All other chemicals were of analytical grade.

Assay of drug-adsorbent interaction

As a rule, a batch method was used: an aqueous solution of the drug was mixed with the adsorbent under experimental conditions indicated in the text. The incubation mixture was shaken at 25°C for minimally 10 min. After the incubation, the adsorbent was separated from the aqueous bulk phase by sedimentation through a mild centrifugation procedure ($1000 \times g$, 10 min). The supernatant was assayed for the amount of drug not bound to the adsorbent.

The interaction of vincristine, methotrexate and cytarabine with Dowex was assayed with a minicolumn (2.0 × 1.5 cm). A 5-ml plastic syringe with a polyethylene disk in the barrel was filled with Dowex. The column was inserted into a test tube so that it was supported at the top of the tube by the finger grips of the syringe. After spinning the column at $1000 \times g$ for 5 min to remove excess buffer, the drug solution was applied to the Dowex column. The column was spun again at $1000 \times g$ for 5 min. Radioactivity of the ^{3}H-labelled drug solutions was determined before and after Dowex treatment to calculate the percentage of bound drug.

Also in the case of doxorubicin-Sephadex interaction, a column method was used: under experimental conditions indicated in the text, an aqueous solution of doxorubicin was applied to a resin column, which had previously been equilibrated with the same aqueous phase without doxorubicin. The eluate was assayed for doxorubicin not bound to the resin.

Analytical assays of the cytostatic drugs

Doxorubicin was routinely assayed spectrophotometrically at 480 nm, cisplatin by means of atomic absorption spectrophotometry. Cytarabine, methotrexate and vincristine were radioassayed by adding trace amounts of [^{3}H]cytarabine, [^{3}H]-methotrexate and [^{3}H]vincristine to the respective drug solutions.

For monitoring the chemical stability of doxorubicin, a reversed phase high performance liquid chromatographic (HPLC) technique as described in Ref. 31 was used. Briefly, the HPLC system consisted of a solvent delivery system Model M-45, a U6K injection device (both from Waters Associates, Milford, MA, U.S.A.) and a Perkin-

Elmer LS-1 Fluorescence Detector (Perkin-Elmer Ltd., Beaconsfield, U.K.). The analytical column (30 cm × 3.9 mm i.d.) was filled with Lichrosorb RP8 (10 μm particles) packing material (Merck). Doxorubicin was chromatographed with a mobile phase composed of 50% (v/v) acetonitrile and 50% (v/v) water. The pH of this eluent was adjusted to 2 with perchloric acid. A flow rate of 1.5 ml/min was employed. The detection was performed at excitation wavelength 480 nm and emission wavelength 550 nm.

Preparation of liposomes

Doxorubicin-containing liposomes. A chloroform/methanol mixture (1 : 1, v/v) containing both doxorubicin and the desired lipids (PC, PS and cholesterol) was evaporated to dryness on a rotary vaporizer under reduced pressure at 40–45°C. The lipid-drug film was evacuated for at least 2 h. Subsequently, glas beads and the hydration medium (145 mM NaCl, 10 mM Tris-HCl, pH 4) were added. Nitrogen was passed through the hydration buffer for about 15 min. The film was hydrated by handshaking at 45°C and left after complete dispersion in a refrigerator for one night. At this stage of preparation one ml of the dispersion contained about 40 μmol phospholipid (PL) and 2.5 mg doxorubicin.

The liposomes, mainly multilamellar vesicles (MLV), were sized by extrusion successively through polycarbonate membrane filters with pore diameters of 0.6 and 0.2 μm (Uni-pore, Bio-Rad, Richmond, CA) under nitrogen pressures up to 0.8 MPa.

Free (non-liposome-associated) doxorubicin was removed by mixing the dispersion with Dowex (sodium form) for 5 min (minimally 1 g Dowex/1 ml dispersion). The cation exchange resin was separated from the liposome containing supernatant by filtration through 8.0 μm membrane filters (Uni-pore, Bio-Rad, Richmond, CA). Less than 10% of doxorubicin was in the free form after the Dowex treatment. No pH change occurred.

In the case of negatively charged liposomes, the amount of liposome-associated doxorubicin was determined by separating free doxorubicin from liposome-bound doxorubicin by gel filtration (Sephadex G-50 fine, Pharmacia, Sweden). In the case of neutral liposomes, the separation was achieved by ultracentrifugation for 1 h at $10^5 \times g$. Then, after destruction of the liposomes by addition of Triton X-100, doxorubicin was quantitatively determined. Lipid phosphorus was determined according to the procedure of Fiske and SubbaRow [19]. Full details on the preparation and characterization of doxorubicin-containing liposomes are reported elsewhere [20,21].

Cisplatin-containing liposomes. Their preparation procedure was nearly identical to that described for the doxorubicin-containing liposomes. Cisplatin was dissolved in the hydration medium (35 mM NaCl, 230 mM mannitol, pH 4). 4 g Dowex/ml dispersion was used to remove free cisplatin from the suspension after extrusion.

Vincristine-containing liposomes. The preparation procedure of these liposomes was somewhat different from the procedures described above. Vincristine was added during the hydration step (hydration medium: 10 mM acetate, 145 mM NaCl, pH 4.5; vincristine 1 mg/ml). The lipid concentration amounted to 10 μmol/ml. The liposomes were probe sonicated for 1 h on ice to obtain small unilamellar vesicles. Free vincristine in the liposome suspension was removed using a Dowex minicolumn containing 2.5 g Dowex (see 'Assay of drug-adsorbent interaction'). This procedure had to be performed twice. No pH change occurred.

Zeta-potential determinations

The electrophoretic mobility of both adsorbents and liposomes was measured with a Mark II microelectrophoresis apparatus (Rank Bros., Cambridge, U.K.). Zeta potentials were calculated from averaged mobilities of at least 20 particles in both directions. Care was taken to focus on the stationary levels. As a rule, mobilities were determined at both the upper and the lower stationary level. The experimental temperature was 25°C.

Conductivity determinations

The specific conductivity (κ) was measured with a Philips PW 9505 conductometer. The cell constant was obtained with KCl solutions with a known specific conductivity at 25°C.

TABLE I

EXPERIMENTAL CONDITIONS TO ESTABLISH THE INTERACTION OF A NUMBER OF CYTOSTATICS WITH DOWEX

All drugs were bound for over 95% by Dowex. Ratio drug to Dowex used: 1 mg/ml (vincristine, doxorubicin, cytarabine, methotrexate), 0.3 mg/ml (cisplatin).

Cytostatic	pH aqueous medium	Net charge	Ref.
Vincristine	4.5 [a]	+	22
	7.2 [b]	+	22
Doxorubicin	4.0 [c]	+	22,23
	8.6 [c]	0	22,24
	10.0 [c]	−	22,24
Cisplatin	4.0 [c]	0	25
	7.4 [c]	0	25
Cytarabine	4.5 [a]	0	22
	7.2 [b]	0	22
Methotrexate	4.5 [a]	0/−	22,26
	7.2 [b]	−	22,26

[a] 10 mM acetate, 145 mM NaCl.
[b] 10 mM phosphate, 145 mM NaCl.
[c] 10 mM Tris, 145 mM NaCl.

Results and Discussion

Binding of cytostatic drugs to Dowex

All the cytostatic drugs under investigation were bound for over 95% by Dowex. Table I summarizes the experimental conditions. As expected the positively charged vincristine and doxorubicin (pH 4) ions strongly interacted with Dowex. Surprisingly, also non-positively charged drugs were bound to the same extent. Under the experimental conditions methotrexate and cytarabine were negatively charged and neutral, respectively [22,26]. At pH 8.6 doxorubicin has no net charge [22,24]. It has been reported that the presence of NaCl produced a pronounced enhancement of stability of cisplatin against hydrolytic conversion to cationic aquation products. This stabilizing effect increased with increasing sodium chloride concentration up to 0.9% [25]. Therefore, a neutral cisplatin molecule was expected to exist in the solutions. The results presented in Table I indicate that apart from electrostatic interactions other binding mechanisms might be active.

Table II shows that neutral or negatively charged liposomes loaded with cytostatics with widely varying molecular structure did not interact with Dowex. The non-encapsulated drug fraction was effectively and rapidly (within a few minutes) removed from the dispersion, while the liposome recovery with respect to both phosphate and encapsulated cytostatic after Dowex treatment ap-

TABLE II

EFFICIENCY OF DOWEX TREATMENT FOR REMOVAL OF NON-ENCAPSULATED CYTOSTATICS FROM LIPOSOME DISPERSIONS

Values presented are means of duplicate experiments. Chol, cholesterol. The Dowex treatment-induced loss of encapsulated cytostatic and phosphate was below 10% in each case. The non-encapsulated cytostatic present after Dowex treatment, expressed as percentage of total amount of cytostatic present in the liposome dispersion after Dowex treatment, was below 10% in each case. (It was necessary to subject the dispersion containing vincristine to Dowex treatment twice.). n.d., not determined.

Liposome composition		Encapsulated cytostatic	pH hydration medium	Zeta potential (mV) [a]	Encapsulation efficiency (%) [b]
PC:Chol	10:4	doxorubicin	4.0 [c]	0	15
PC:PS:Chol	10:1:4	doxorubicin	4.0 [c]	−10	32
PC:PS:Chol	10:1:4	cisplatin	4.0 [d]	−22	11
PC:PS:Chol	10:1:7.5	vincristine	4.5 [e]	n.d.	4

[a] For all individual dispersions the coefficients of variation of the velocities measured at the two stationary levels for both directions were less than 10%.
[b] Percentage of the drug initially added to the preparation that becomes liposome-associated under the experimental conditions mentioned in Materials and Methods.
[c] 10 mM Tris, 145 mM NaCl.
[d] 230 mM mannitol, 35 mM NaCl.
[e] 10 mM acetate, 145 mM NaCl.

proached 100%. These data indicate that the Dowex procedure allows for a rapid, efficient and convenient method for the removal of non-entrapped drugs from liposome dispersions with no dilution of the external aqueous phase. Studies with liposome-encapsulated drugs will be facilitated by the use of this method, since it avoids many of the problems introduced by current procedures as dialysis, gelfiltration and centrifugation/washing.

Binding of doxorubicin to different adsorbents

A number of adsorbents was selected to study their doxorubicin binding capacities in aqueous media. Prior to doxorubicin-adsorption assays the zeta-potentials were determined. This parameter provides information on the probability of an electrostatic contribution to the drug-adsorbent interaction. The results of measurements of the electrophoretic mobility of various adsorbents are presented in Table III.

Altering the zeta-potential from negative to zero (Silica gel) or to positive (aluminium oxide) by lowering the pH reduced the interactions. Drug release was induced by increasing the ionic strength (Silica gel, Sephadex). Negative molecules of doxorubicin (pH 10) did not adsorb onto the cation exchange resin Sephadex. Based on these results it is concluded that for Sephadex, Silica gel and aluminium oxide doxorubicin retention was mainly based on electrostatic interactions.

Because Amberlite does not carry ion exchange active groups attached to the polystyrene matrix like Dowex, the adsorption forces in the

TABLE III

ZETA POTENTIAL AND DOXORUBICIN BINDING PROPERTIES OF A NUMBER OF ADSORBENTS UNDER VARYING CONDITIONS

Adsorbent	Aqueous medium	Zeta potential		Binding properties [a]
		mV	coefficient of variation (%)	
Silica gel	demineralized H_2O	−33	13	+
	10 mM Tris, 145 mM NaCl, pH 4.0	−10	37	−
	0.1 M HCl	0	0	−
Aluminium oxide	10 mM Tris, 145 mM NaCl, pH 9.2	−23	30	+
	10 mM Tris, 145 mM NaCl, pH 4.0	+13	49	−
Sephadex	demineralized H_2O	−63	10	+ [c]
	10 mM Tris, 145 mM NaCl, pH 4.0	−30	17	−
	10 mM Tris, 145 mM NaCl, pH 10.0	−33	11	−
	1.7 M NaCl	n.d. [f]		−
Amberlite [b]	demineralized H_2O	−21	14	+ [d]
	10 mM Tris, 145 mM NaCl, pH 4.0	0	0	+ [d]
Dowex [b]	demineralized H_2O	−35	17	+ [e]
	10 mM Tris, 145 mM NaCl, pH 4.0	−23	14	+ [e]
	10 mM Tris, 145 mM NaCl, pH 8.6	−24	13	+ [e]
	10 mM Tris, 145 mM NaCl, pH 10.0	−25	16	+ [e]
	1.7 M NaCl	n.d. [f]		+ [e]

[a] +: less than 5% of doxorubicin left in the supernatant after the doxorubicin-adsorbent incubation. −: more than 95% of doxorubicin left in the supernatant after the doxorubicin-adsorbent incubation.
In case of positive binding properties, possible gel-filtration effects were minimized by extensive washing of the adsorbent with the aqueous phase without doxorubicin.
The ratio doxorubicin to adsorbent used was minimally 2 mg/g.
[b] The adsorbent was ground in a ball mill to obtain particle sizes suitable for zeta-potential measurements.
[c] No desorption by washing with methanol and ethanol. Desorption with 1.7 M NaCl.
[d] No desorption by raising the pH to 10 or by washing with 1.7 M NaCl and 4 M NaOH. Desorption with methanol and ethanol.
[e] No desorption by washing with 1.7 M NaCl, 0.1 M HCl, methanol, ethanol, chloroform, 2% cetrimide, 2% benzalkonium chloride. Desorption with NaOH solutions with pH > 11.5.
[f] n.d., not determined.

doxorubicin-Amberlite complex are supposed to be primarily of the Van der Waals type. The following observations indicated the hydrophobic nature of this interaction: (1) drug binding occurred in the absence of a net zeta potential and charge reversal of doxorubicin by increasing the pH from 4 to 10 did not affect the interaction; (2) no change in interaction was observed over a wide range of ionic strengths; (3) the drug was released from Amberlite by washing with methanol or ethanol. With Dowex the interaction mechanism between doxorubicin and the resin was more complex. In contrast to Sephadex this type of cation exchange resin did adsorb doxorubicin ions with a negative charge (pH 10). The drug-adsorbent binding forces were resistant to large, positively charged amphiphilic ions (cetrimide, benzalkonium chloride) competing for the binding sites. These cations could not displace doxorubicin from the resin. A low pH (0.1 M HCl), high ionic strength (10% NaCl), methanol and chloroform extraction all failed to liberate doxorubicin. Only with NaOH solutions with pH values over 11.5 the drug was released. The drug and the resin were only briefly exposed to these extreme pH conditions to avoid any chemical degradation of doxorubicin. Acidification of this eluate resulted in renewed binding to Dowex. This finding indicates that only near the pK_a for the second deprotonation of the ring structure sufficient repulsive forces were mobilized to remove doxorubicin from the resin [23].

On the basis of these results, it was concluded that in contrast to the (mainly) electrostatic interaction of doxorubicin with Sephadex (a strongly acidic cation exchanger with a hydrophylic, dextran-based matrix) and the hydrophobic interaction with amberlite (a polystyrene-based resin consisting of a hydrophobic matrix which does not contain ion exchange active groups), the interaction of doxorubicin with Dowex (composed of both a hydrophobic, Amberlite-like matrix and exchangeable functional groups) involves apart from electrostatic also hydrophobic components.

Mode of adsorption of doxorubicin onto Dowex

In order to further clarify the nature of the doxorubicin-Dowex interaction, additional studies were undertaken on the electrostatic and hydrophobic components of the binding mechanism.

To establish the contribution of electrostatic interactions to the doxorubicin-Dowex adsorption process, the conductivity of doxorubicin solutions (demineralized water) was measured before and after the addition of Dowex, Sephadex and Amberlite. The results are presented in Fig. 1. Doxorubicin was bound by all three adsorbents used; no doxorubicin could be detected in the aqueous 'bulk' phase. After addition of Dowex or Sephadex, the conductivity increased and almost coincided with the sodium chloride 'calibration' curve. If sodium ions were quantitatively exchanged for doxorubicin ions with a stoichiometry of 1 : 1 the slopes of the curves after adsorbent addition should equal the sodium chloride 'calibration' curve. The observation that binding of cationic doxorubicin to Dowex had no effect on the negative zeta potential (Table III: -23 mV) also suggested the occurrence of ion exchange in the doxorubicin-Dowex adsorption process. The molar conductivity of sodium chloride calculated from the sodium chloride calibration curve (12.7 $mS \cdot m^2 \cdot mol^{-1}$) compared well with literature data (12.64 $mS \cdot m^2 \cdot mol^{-1}$) [27]. The data points representing the specific conductivity of doxorubicin solutions depended linearly on the concentration of the drug suggesting that under the experimental conditions no self-association of doxorubicin occurred. On the basis of doxorubicin-hydrochloride specific conductivity data from both the Dowex and Sephadex experiments the molar conductivity of the doxorubicin cation is calculated to be approximately 2.5 $mS \cdot m^2 \cdot mol^{-1}$ (molar conductivities of the sodium and chloride ions taken from literature) [27]. Now, assuming the doxorubicin cation to be spherical, a rough approximation of the dimensions of the doxorubicin cation can be obtained by using Stokes' law. A diameter of 0.33 nm is calculated [27,28].

Addition of Amberlite to doxorubicin solutions resulted in a complete removal of the drug from the aqueous phase. The specific conductivity of the aqueous phase after binding did not significantly deviate from the specific conductivity of demineralized water (around 0.2 $mS \cdot m^{-1}$). This finding is strongly different from the results obtained with Dowex and Sephadex and concurs with suggestions made above: the occurrence of electrostatic interactions in case of Dowex and

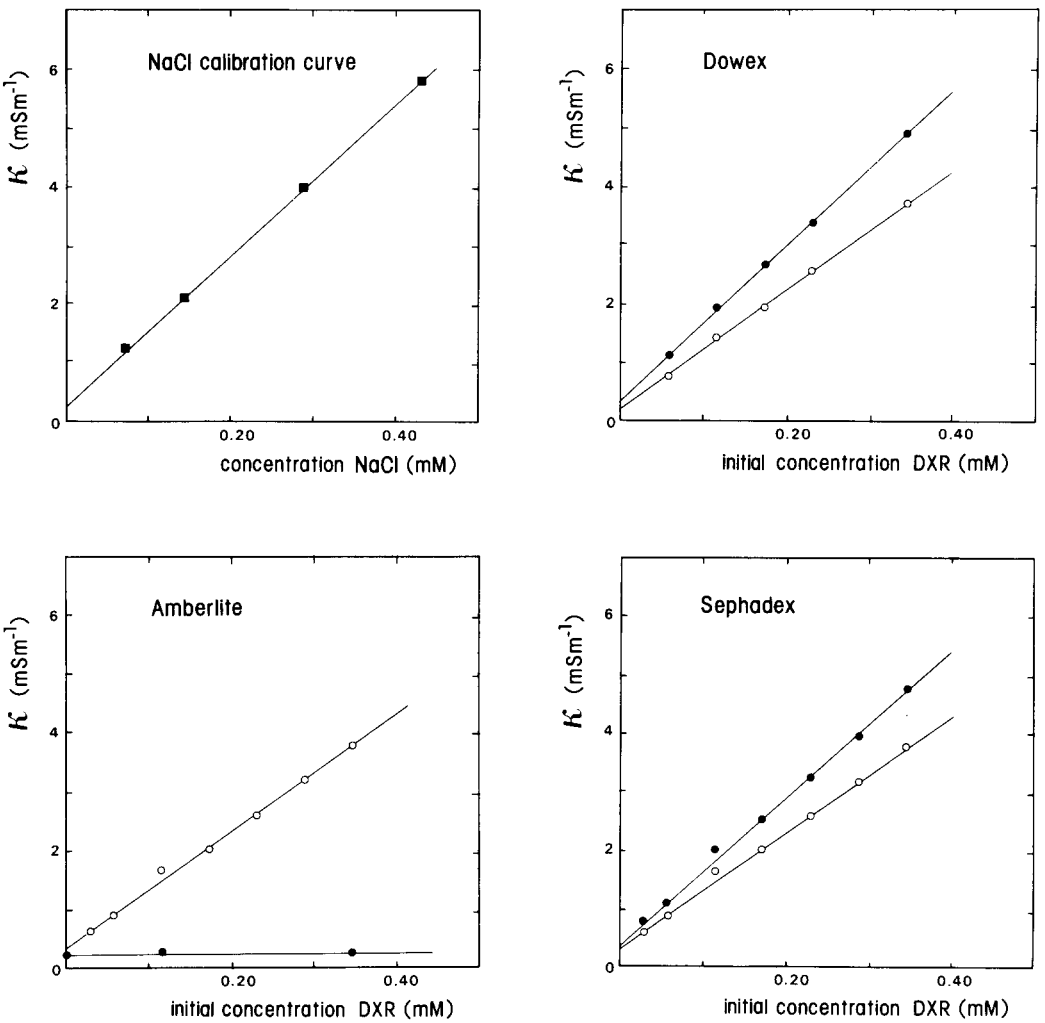

Fig. 1. The specific conductivity (κ) of doxorubicin solutions (demineralized water) before (○) and after (●) the addition of different adsorbents. NaCl calibration curve (■): slope 12.69 $mS \cdot m^2 \cdot mol^{-1}$. Dowex: slope before addition (○) 10.04 $mS \cdot m^2 \cdot mol^{-1}$; slope after addition (●) 13.25 $mS \cdot m^2 \cdot mol^{-1}$. Sephadex: slope before addition (○) 10.01 $mS \cdot m^2 \cdot mol^{-1}$; slope after addition (●) 12.71 $mS \cdot m^2 \cdot mol^{-1}$. Amberlite: slope before addition (○) 10.06 $mS \cdot m^2 \cdot mol^{-1}$. Lines are calculated according to linear regression analysis. Addition of the adsorbents did not change the pH. κ for pure demineralized water: about 0.2 $mS \cdot m^{-1}$. Experimental temperature, 25°C. DXR, doxorubicin.

Sephadex and the absence of these interactions when Amberlite was used as an adsorbent.

Studies concerning the temperature dependence of doxorubicin adsorption onto the adsorbents show an adsorbent-dependent behaviour. Results are given in Table IV.

A thermodynamic characteristic of an electrostatically controlled interaction is a negative enthalpy change (ΔH) [29]. Using the Van 't Hoff isochore [30], these experiments pointed out that only the temperature dependence of doxorubicin adsorption onto Sephadex corresponded with a negative ΔH. In that case doxorubicin adsorption was reversible: desorption with increasing temperature followed by readsorption on cooling. No reversible doxorubicin desorption was observed for Dowex ($\Delta H \geq 0$). With Amberlite no temperature dependence at all was observed. These findings provided additional evidence for the important role of hydrophobic effects in the

TABLE IV

ADSORBED AMOUNTS OF DOXORUBICIN ONTO VARIOUS ADSORBENTS AT DIFFERENT TEMPERATURES

Doxorubicin-adsorbent complexes were prepared at 25°C by incubating appropriate amounts of adsorbent with aqueous doxorubicin solutions (10 mM Tris, 145 mM NaCl, pH 4.0). After equilibrating for 4 days, the temperature was raised to 50°C and kept at this temperature for 2 h. Subsequently, the temperature was elevated to 70°C and kept at this temperature for another 2 h. Finally, the temperature was lowered to and kept at 25°C. During the experiment, the dispersion was well stirred. No pH change occurred. No doxorubicin degradation could be monitored with an HPLC method [31].

Adsorbent	Adsorbed amount of doxorubicin (mequiv. doxorubicin/g adsorbent) [a]						
	25°C	[b] →	50°C	→	70°C	→	25°C
Sephadex	1.22	1.00	0.71	1.14			
Amberlite	0.16	0.16	0.16	0.17			
Dowex	0.52	0.58	0.72	0.75			

[a] Adsorbed amounts were quantified as described in Materials and Methods.
[b] →: temperature change.

doxorubicin-Dowex interaction mechanism. Short range hydrophobic (Van der Waal's) forces in combination with electrostatic forces are responsible for the strong affinity of doxorubicin for Dowex as reflected by the high resistance of the binding forces against desorption of bound doxorubicin (Table III).

Concluding remarks

This report showed that cytostatic drugs widely varying in their molecular structure can be removed from aqueous solutions by Dowex. Neutral liposomes or liposomes with a negative zeta potential do not interact with Dowex. Therefore, this ion exchange resin can be used for a rapid, efficient and convenient removal of non-entrapped drugs from liposome dispersions. The binding of doxorubicin to Dowex involves apart from electrostatic also hydrophobic components. The combination of these probably accounts for the extremely high affinity of doxorubicin for Dowex. For other adsorbents the interactions with doxorubicin are either mainly of an electrostatic (Sephadex, Silica gel and aluminium oxide) or a hydrophobic (Amberlite) nature. As a result of their binding characteristics (Table III), aluminium oxide and Silicagel are unsuitable to be used as free doxorubicin adsorbing materials in the preparation of doxorubicin containing liposomes. On the other hand, Sephadex and Amberlite seem to be useful alternatives for Dowex. However, application of these adsorbents exhibits several drawbacks: use of Sephadex causes dilution of the liposomal preparation (a gel filtration column method has to be used), use of Amberlite results in a less effective removal system as compared to Dowex because of the relatively low doxorubicin binding capacity (Table IV).

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References

1 Mayhew, E. (1983) in Liposomes (Ostro, M.J., ed.), pp. 289–341, Marcel Dekker, Inc., New York and Basel
2 Yatvin, M.B. and Lelkes, P.I. (1982) Med. Phys. 9, 149–175
3 Weinstein, J.N. (1981) Pure Appl. Chem. 53, 2241–2254
4 Coune, A. (1984) Eur. J. Cancer Clin. Oncol. 20, 443–445
5 Szoka, F. and Papahadjopoulos, D. (1980) Annu. Rev. Biophys. Bioeng. 9, 467–508
6 Klingenberg, M. (1970) in Essays in Biochemistry (Campbell, P.N. and Dickens, F., eds.), Vol. 6, pp. 119–159, Academic Press, New York

7 Moro, L.C.V., Neri, G. and Rigamonti, A., Deutches Patentamt A 61 K 9/00
8 Van Bommel, E.M.G. and Crommelin, D.J.A. (1984) Int. J. Pharm. 22, 299–310
9 Freise, J., Mueller, W.H., Magerstedt, P. and Schmoll, H.G. (1982) Arch. Int. Pharmacodyn. 258, 180–192
10 Sur, B., Ray, R.R., Sur, P. and Roy, D.K. (1983) Oncology 40, 372–376
11 Juliano, R.L. and McCullough, H.N. (1980) J. Pharmacol. Exp. Ther. 214, 381–387
12 Ganapathi, R., Krishan, A., Wodinsky, I., Zubrod, C.G. and Lesko, L.J. (1980) Cancer Res. 40, 630–633
13 Van Hoesel, Q.G.C.M., Steerenberg, P.A., Crommelin, D.J.A., Van Dijk, A., Van Oort, W., Klein, S., Douze, J.M.C., De Wildt, D.J. and Hillen, F.C. (1984) Cancer Res. 44, 3698–3705
14 Rahman, A., More, N. and Schein, P.S. (1982) Cancer Res. 42, 1817–1825
15 Freise, J., Magerstedt, P. and Schmidt, F.W. (1979) Z. Naturforsch. Sect. C. Biosci. 34, 114–119
16 Kaye, S.B., Boden, J.A. and Ryman, B.E. (1981) Eur. J. Cancer 17, 279–289
17 Kirby, C. and Gregoriadis, G. (1983) Biochem. Pharmacol. 32, 609–615
18 Firth, G., Oliver, A.S. and McKeran, R.O. (1984) J. Neurol. Sci. 63, 153–165
19 Fiske, C.H. and SubbaRow, Y. (1925) J. Biol. Chem. 66, 375–400
20 Crommelin, D.J.A., Slaats, N. and Van Bloois, L. (1983) Int. J. Pharm. 16, 79–92
21 Crommelin, D.J.A. and Van Bloois, L. (1983) Int. J. Pharm. 17, 135–144
22 Martindale (1982) 28th Edn. (Reynolds, J.E.F., ed.), The Pharmaceutical Press, London
23 Sturgeon, R.J. and Schulman, S.G. (1977) J. Pharm. Sci. 66, 958–961
24 Eksborg, S. (1978) J. Pharm. Sci. 67, 782–785
25 Atilla Hincal, A., Long, D.F. and Repta, A.J. (1979) J. Parent. Drug Assoc. 33, 107–116
26 Hansen, J., Krielgard, B., Nielsen, O. and Jorgen, V. (1983) Int. J. Pharm. 16, 141–152
27 Wallwork, S.C. and Grant, D.J.W. (1977) Physical Chemistry , pp. 348–352, 3rd Edn., Longman Group Ltd, London
28 Hiemenz, P.C. (1977) Principles of Colloid and Surface Chemistry, pp. 454–455, Marcel Dekker, Inc., New York and Basel
29 Timasheff, S.N. (1972) in Protides of the Biological Fluids (Peeters, H., ed.), pp. 511–519, Pergamon Press, Oxford
30 Hiemenz, P.C. (1977) Principles of Colloid and Surface Chemistry, p. 320, Marcel Dekker, Inc., New York and Basel
31 Beijnen, J.H., Wiese, G. and Underberg, W.J.M. (1985) Pharm. Weekbl. Sci. Edn. 7, 109–116