ELSEVIER

Journal of Chromatography B, 702 (1997) 181–191

JOURNAL OF CHROMATOGRAPHY B

On the use of ion-pair chromatography to elucidate doxorubicin release mechanism from polyalkylcyanoacrylate nanoparticles at the cellular level

Xavier Pepin[a], Loïc Attali[a], Catherine Domrault[a], Sacha Gallet[a], Jean M. Metreau[a], Yannick Reault[a], Philippe J.P. Cardot[a,*], Mohamed Imalalen[b], Catherine Dubernet[c], Emilienne Soma[c], Patrick Couvreur[c]

[a]*Laboratoire de Chimie Analytique et d'Electrochimie Organiques, Faculté de Pharmacie, Université Paris Sud, 5 Rue J.B. Clément, F 92296 Châtenay Malabry Cedex, France*

[b]*Laboratoire de Synthèse Organique, Faculté de Pharmacie, Université Paris Sud, 5 Rue J.B. Clément, F 92296 Châtenay Malabry Cedex, France*

[c]*Laboratoire de Physicochimie, Pharmacotechnie et Biopharmacie, URA CNRS 1218, Faculté de Pharmacie, Université Paris Sud, 5 Rue J.B. Clément, F 92296 Châtenay Malabry Cedex, France*

Received 22 January 1997; received in revised form 18 June 1997; accepted 4 July 1997

Abstract

The major hypothesis underlying the remarkable efficiency of polyalkylcyanoacrylate particles loaded with doxorubicin against multidrug resistant tumor cells in vitro, is based on the ion-pair association of doxorubicin with soluble hydrolysis products of polyalkylcyanoacrylate. In an attempt to demonstrate the validity of this hypothesis, we have used ion-pair reversed-phase high-performance liquid chromatography and a laboratory-synthetized compound, i.e., the 2-cyano-2-butylhexanoic acid, as a model for polyalkylcyanoacrylate highly polydispersed degradation products. It is shown that, compared to a counter-ion, like heptane sulfonic acid, 2-cyano-2-butylhexanoic acid exhibits an effective ion-pairing effect at different pH values and organic mobile phase conditions. Moreover, at pH close to physiological conditions and at low mobile phase organic modifier percentage, this effect is experimentally observed, which strongly supports the initial hypothesis. © 1997 Elsevier Science B.V.

Keywords: Polyalkylcyanoacrylate nanoparticles; Doxorubicin

1. Introduction

Doxorubicin is an antracyclin-type cytotoxic antibiotic mainly used in the treatment of leukemia and of solid tumors. The use of doxorubicin solutions in cancer chemotherapy is restricted by the cardiotoxicity of the drug, and by the appearance of tumor resistance to doxorubicin, especially in multidrug resistance (MDR). In aqueous medium, dissolved doxorubicin tends to form self associating complexes leading to dimers and tetramers [1]. The doxorubicin molecule is usually considered to be a polybase system, and the most basic state is assigned to the

*Corresponding author. Present address: Laboratoire de Chimie Analytique et de Bromatologie, Faculté de Pharmacie, Université de Limoges, 2 Rue du Dr. Marcland, F87025 Limoges Cedex, France.

0378-4347/97/$17.00
PII S0378-4347(97)00362-9

monomeric form [1]. The use of colloidal suspensions of nanospheres loaded with doxorubicin has led to a considerable reduction of the drug cardiotoxicity [2] and to a substantial enhancement of its efficiency against hepatic metastasis [3]. Additionally, in cases of MDR, polyalkylcyanoacrylate (PACA) doxorubicin loaded nanoparticles were reported to overcome the resistance of various tumor cell lines in culture [4–6]. MDR is due to the overexpression of a membrane protein, P-glycoprotein (Pgp), which is supposed to pump the drug out of the cells, leading to the restriction of cytotoxic effect [7]. Nanospheres were therefore employed with, the idea of protecting the doxorubicin from the excretion action of Pgp, as it enters the cells by endocytosis [8]. Recently, it was demonstrated that PACA nanospheres are not endocytosed by tumor cells [9]. Instead, they are adsorbed by the cell membrane, delivering the encapsulated doxorubicin close to it [9].

In a biological medium, PACA nanospheres are bioeroded, thus releasing doxorubicin, polyalkylcyanoacrylic acid and alcohol [10]. Dubernet et al. [11] noted that a complex, consisting of free doxorubicin and soluble polyalkylcyanoacrylic acid, may appear during the bioerosion close to the cell membrane. The complex which is most likely to be formed is an ion-pair made of positively charged doxorubicin and a negatively charged PACA degradation product. The charge masking of doxorubicin leads to a more lipophilic prodrug, as schematically shown in Fig. 1A, which therefore enters the cells to a greater extent than the free drug [12].

An increasing number of reports show that when using nanoparticles, only PACA are able to overcome MDR [6,13]. On the other hand, modification of the sugar moiety of doxorubicin leading to the absence of a positive charge on the molecule, has been reported to overcome MDR [14]. As the theory of charge masking by the formation of an ion-pair proved to be relevant, there was a need to investigate it experimentally. For this purpose, ion-pair reversed-phase high-performance liquid chromatography (RP-HPLC) was chosen [15]. The aim of the study was to determine the retention characteristics of doxorubicin in the presence of PACA degradation compounds, and to compare doxorubicin retention characteristics with either a classical counter-ion or PACA compounds. However, because of a rather high average molecular mass of PACA (≈1000) and a large polydispersity as measured by Vansnick et al. [16], it has not been possible to simply use the polymer resulting from the bioerosion of nanoparticles. A model compound was therefore designed and synthetized. This "polyalkyl cyanoacrylic acid-like" molecule was the 2-cyano-2-butylhexanoic acid (CHX). The synthesis scheme and structure of CHX are given in Fig. 1B. It was obtained with a 99% purity. It has a molecular mass of 197.28 and a measured pK_a of 2.8. This compound seemed to act as a possible counter-ion reagent with HPLC properties analogous to those classical counter-ion reagent used in HPLC with basic compounds, e.g., heptane sulfonic acid (HX), associated with a 220.27 molecular mass. These two compounds behave similarly in terms of hydrophobic queuing.

2. Experimental

2.1. Synthesis and characterisation of 2-cyano-2-butylhexanoic acid (CHX)

CHX synthesis was done in two steps. First, an esterified compound was obtained: ethyl-2-cyano-2-butylhexanoate. Hydrolysis was performed in a second step and led to 2-cyano-2-butylhexanoic acid.

2.1.1. Ethyl 2-cyano-2-butylhexanoate

A solution of ethylcyanoacetate (800882: Lab. Merck–Clevenot, Nogent, France), in ethanol (0.15 *M*) was treated with sodium ethoxide (0.30 *M*; 820871: Lab. Merck–Clevenot) at room temperature (20°C) for 30 min. Butyl bromide (0.30 *M*; 801602: Lab. Merck–Clevenot) was then added for a period of 1 h. The reaction mixture was stirred at room temperature for 2 h. After cooling in an ice-bath, the reaction mixture was neutralized with glacial acetic acid Sigma–Aldrich (St. Quentin Fallavier, France). The precipitate obtained was filtered off, and washed twice with ethanol (Reidel-de Haen, Seelze, Germany). The reaction mixture was then distilled under reduced pressure (0.2 atm; 1 atm=101 325 Pa) to remove ethanol solvent and a yellow, viscous liquid of 0.92 density was obtained. This compound was then analysed, using infrared (IR) and nuclear magnetic resonance (NMR) techniques, and identified as

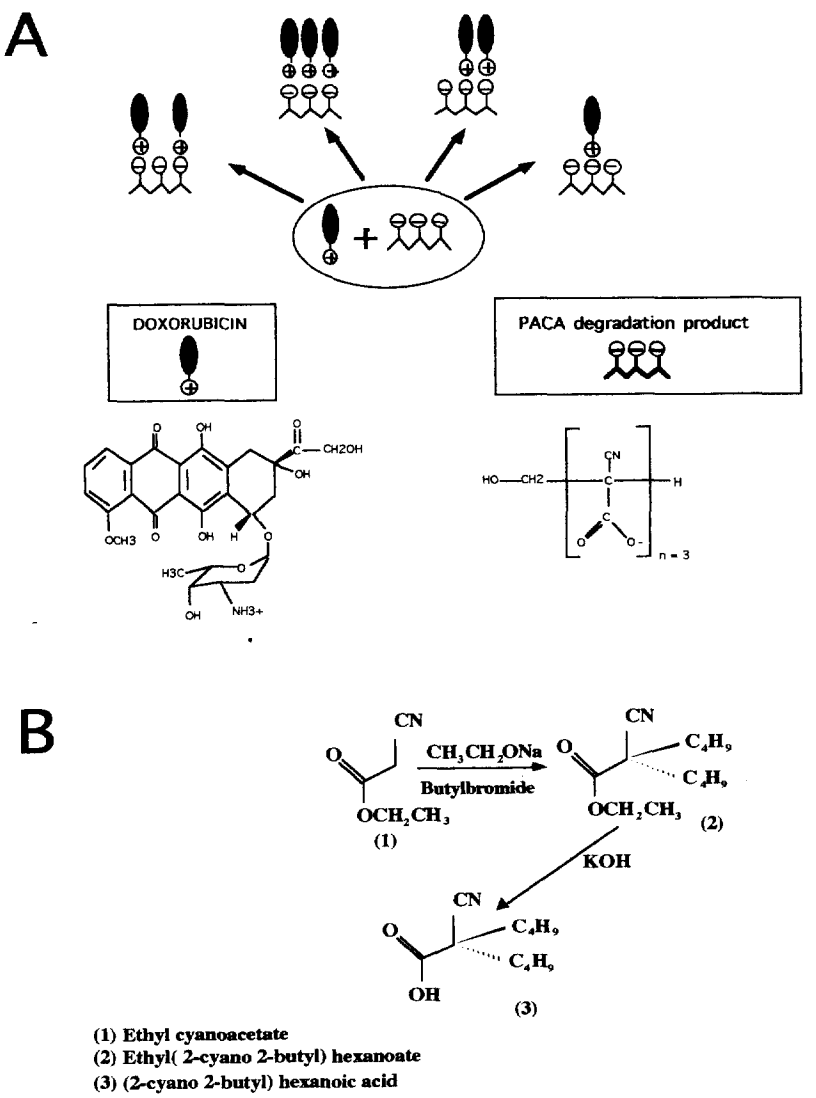

Fig. 1. 2-Cyano-2-butylhexanoic acid and doxorubicin. (A) Hypothesis on the biological action of PACA ion pair formation with doxorubicin. (B) Structure of the hypothetic ion-pair formation and synthesis of the 2-cyano-2-butylhexanoic acid.

ethyl-2-cyano-2-butylhexanoate obtained with a 80% yield.

Structural analysis: IR spectra were obtained using a Perkin-Elmer spectrophotometer Model 297 (St. Quentin en Yvelines, France) and NMR spectra with a Brucker AM-200-SY spectrometer (Brucker, Strasbourg, France).

I.R.: 2960; 2225; 1735; 1210; 1100 cm^{-1}. ^{1}H NMR ($CDCl_3$): 0.9 (6H, t); 1.3 (7H, m); 1.5 (4H, m); 1.8 (4H, m); 4.25 (2H, q). ^{13}C NMR ($CDCl_3$): 13.5 (2q); 13.9 (q); 21.7 (2t); 27.3 (2t); 37 (2t); 49.8 (s); 62.3 (t); 119.3 (s); 169.1 (s).

2.1.2. Synthesis of 2-cyano-2-butylhexanoic acid

Ethyl-2-cyano-2-butylhexanoate was hydrolysed with alcoholic potassium hydroxide (Sigma–Aldrich). The free-acid CHX was obtained after treatment with diluted hydrochloric acid. After extraction with ethyl oxide, the organic phase was dried over magnesium sulfate (Sigma–Aldrich), giving an

orange, viscous liquid (90% yield) with a density of 0.98. A series of organic-phase extractions and dryings was performed to obtain a compound of maximum purity.

2.1.3. Determination of the acidic properties of CHX

This procedure was carried out using two methods. The first employs calculation procedures developed by Perrin et al. [17], referring to aliphatic acids. With this method, pK_a of CHX was calculated to be 2.8 at 25°C, in an aqueous medium of null ionic strength. The second method was a potentiometric titration. A glass electrode was carefully calibrated in aqueous medium using five different standard buffers pH 1, 3, 5, 7, 9 from (OSI, Elancourt, France). Potentiometric titration with 0.10 *M* sodium hydroxide was then performed with 200 μl of CHX diluted in a water–methanol (40:60, w/w) mixture. In this medium the pK_a was found to be 3.4. The use of classical solvent transfer corrections (linked to solvent permittivity) led to a pK_a value of 2.6 in pure water (25°C), that is with a null ionic strength [18]. This pK_a determination clearly showed that actual CHX pK_a ranged between 2.6 and 2.8, indicating that ion-pair associations are possible with CHX and basic compounds on a mechanism analogous to the one encountered for basic compounds with classical HPLC counter-ion HX.

2.2. The chromatographic system

A Model 5000 Varian Liquid Chromatograph (Varian, Les Ulis, France) with a Schoeffel Spectroflow Monitor Model 770 (Cunow, Osny, France) set at 494 nm and a Rheodyne Model 7525 (Rheodyne, Cotati, CA, USA) injection device with a sample loop of 50 μl were used. The detector signal was recorded on a KIPP and Zonen (Touzard et Matignon, Les Ulis, France) Model BD40 strip chart recorder. The separation column from Shandon HPLC (Astmoor, UK), 5 μm Nucleosil C_{18} (150× 4.6 mm I.D.) was inserted between the injection device and the detector. All the experiments described in this report were carried out with the same column and the elutions were isocratic. In order to obtain reliable retention data of doxorubicin under various chromatographic conditions, special attention was paid to the void volume determination and to the mobile phase preparations.

2.2.1. Void volume

Void volume determination experiments were systematically performed using pure water, acetonitrile or methanol injected as samples into the various mobile phases prior to doxorubicin retention analysis. These injections produced a negative and positive peak signal, the gravity center of which was determined as the void volume of the chromatographic system. The mean void volume obtained for this system was 1.05 ± 0.06 ml ($n = 3 \times 12$, 2σ). For each mobile phase composition, a series of measurements of pure organic solvent (acetonitrile, methanol) and pure water were carried out to define the void volume of the system. Depending on the percentage of organic solvent percentage, the measured void volume differed slightly, therefore retention characteristics where described using retention factor values. For doxorubicin, the peak apex was used for retention volume calculations. Therefore void, and retention volumes being experimentally determined, retention factors (k) were easily calculated. Throughout all experiments $\sigma(\log k)$ values were systematically lower than 0.013.

2.2.2. Mobile phases

Mobile phases were prepared by mixing buffers prepared in pure aqueous medium with pH control and organic modifiers. The HPLC grade aqueous medium were prepared with fresh double-distilled water and organic solvents (Methanol Chromasolv, Reidel-de Haen). Ionic strength of the buffer was systematically calculated. For example, citrate buffer (pH measured at 2.2) was made with citric acid (20.68 g; Sigma–Aldrich), NaCl (4.05 g; Sigma–Aldrich), 0.10 *M* NaOH (109.5 ml; Sigma–Aldrich) and bidistilled water up to 1 l, and hence had an ionic strength of $8.032 \cdot 10^{-2}$ *M*; Tris buffer (pH measured at 7.2) made of Tris (11.76 g; Sigma–Aldrich), 0.10 *M* HCl (669.3 ml; Merck–Clevenot) and bidistilled water up to 1 l was also associated with an ionic strength of $8.032 \cdot 10^{-2}$ *M*. Citrate buffer pH 3, acetate buffers pH 3.8 and pH 4.5, phosphate buffer pH 5.4 were prepared in a similar fashion with Sigma–Aldrich reagents. When needed, additional components ($2.54 \cdot 10^{-3}$ *M* triethylamine,

CHX or HX) were added afterwards. The mobile phase was then prepared by mixing appropriate volumes. Mobile phase volume alterations due to mixture process among the organic phase (methanol) and the aqueous one were neglected. In a final step, NaCl (Sigma–Aldrich) was added to keep every mixture at a constant ionic strength of 0.05±0.002 *M*. It is obvious that the ionic strength of these mobile phases can be considered to be far higher than ionic strength classically used in HPLC, but these high values allowed the use of a wide set of buffer types and of pH. It also helped the elution of doxorubicin samples using ionic strengths analogous to those encountered in biological media.

2.2.3. Sample preparation and detection

Concentrated aqueous solutions of doxorubicin (1 mg/ml) were prepared and diluted prior to use in the mobile phase to obtain a 0.05 mg/ml solution. As spectrophotometry of doxorubicin solution showed two maxima at 233 nm and 494 nm, the latter was chosen to avoid solvent interferences. Detection sensitivity was adjusted either to 0.01 or 0.02 a.u.f.s.. The recorder speed was 60 mm/min to obtain reliable and precise measurements. The mobile phase flow-rate was set at 1.0 ml/min and manually controlled during the experiments: the average flow-rate was 1.03±0.02 ml/min.

2.2.4. Elution procedures and measurements for statistical analysis

Mobile phase and column temperatures were kept constant at 20±0.3°C using a thermostatted water bath. For each mobile phase composition used in this report, the column was equilibrated with the mobile phase according to the following procedure: 50 ml of the mobile phase was passed through the column at a low rate (0.2 ml/min). Then, a series of 5 to 7 successive samples of doxorubicin were injected for each 20 ml of mobile phase, the last three were taken into account for retention volume calculations. The retention times were measured during each experiment with an average distribution less than 2%.

The statistical treatment used in this report was needed to compare different mobile phase elution conditions. The pairing data contained doxorubicin log k values versus mobile phase aqueous medium ratio (q). The normality distribution of each series of data (size<30) was first diagnosed with the Shapiro normality test. Then, the series were analysed using two separate and successive procedures. First, a Student's t-test for comparison of mean values comparison was done at a 5% risk. If the mean values of the Student's t-test were not significant, log k curves versus aqueous percentage in the mobile phase were tested by comparing the slope and the origin values according to the method described by Kleibaum and Kupper [19].

3. Results and discussion

Doxorubicin elution characteristics were systematically studied under experimental conditions where pH, buffer systems, solvent strength and mobile phase additives were modified and the ionic strength kept constant. Using simple mobile phases made only of aqueous buffer and organic modifier, retention characteristics were studied for aqueous mixtures containing from 5 to 35% (v/v). Due to the nature and percentage variation of methanol, it can be stated that these mobile phases were aqueous even if the proportion of methanol was high. In Fig. 2, corresponding retention factors k were plotted versus q, which is defined as the ratio of the aqueous mass to the organic mass [for example a water–methanol mixture (65:35, v/v) gave $q=0.538$].

3.1. Dual retention mechanism of doxorubicin

In Fig. 2 a non-linear relationship between log k and the mobile phase proportion of organic modifier was observed, regardless of the elution buffer type or pH. Retention minimum was observed for a q ratio between 0.2 and 0.3, which indicated that this minimum was observed for a volume percentage of aqueous medium comprised between 15 and 25%. At lower q ratio, i.e., a higher methanol proportion in the mobile phase, an increase in log k' indicated that silanol interactions with doxorubicin were operative and played a key role in the retention mechanism [20]. At higher q values, the relatively high percentage of water in the mobile phase competed with doxorubicin for silanol groups, and the systematic increase of log k with the increase in q indicated that the retention was predominantly driven by the sol-

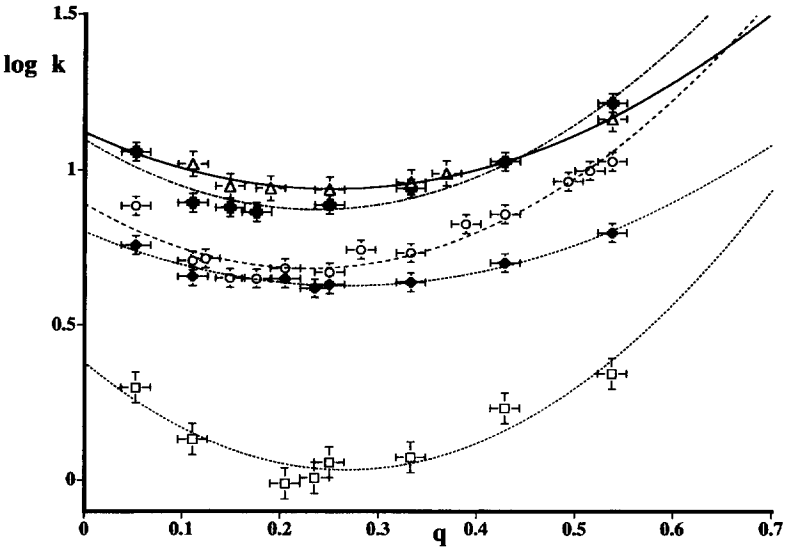

Fig. 2. Effect of mobile phase composition (elution strength, pH, buffer systems) on the combined retention mechanism of doxorubicin. Error bars correspond to $\pm 3\sigma$, variances on abcissa correspond to mixing precision. k i sthe retention factor, q the mobile phase ratio; the mobile phase ionic force is kept constant. For 2nd degree polynomial fitting curves, squared residuals ranged from 0.889 to 0.956. Chromatographic experimental conditions are described in Section 2.2. (□) Citrate buffer, pH 3.0; (♦) acetate buffer, pH 3.8; (○) acetate buffer, pH 4.5; (△) acetate buffer, pH 5.1; (+) phosphate buffer, pH 5.4.

vophobic effect [21]. The experiments reported in Fig. 2 show that, depending on the organic mobile phase percentage, a double elution mechanism in RP-HPLC was operating with a non-negligible adsorption of doxorubicin on the silanol groups at high organic phase percentage. Conversely, at higher water medium percentage a hydrophobic-like retention mechanism could be observed [22]. This interpretation is supported by the 2nd degree polynomial fitting as shown in Fig. 2. Considering the doxorubicin elution characteristics at pH 4.5 where fourteen different mixing proportions of methanol were assayed, a very good accordance is found. Moreover, considering the acetic acid buffer system of different pH, it is observed that log k increased with pH increase, indicating that when the charged percentage of the weak base (the doxorubicin) decreased, retention increased as predicted by the solvophobic theory.

3.2. Conditions for a single retention mechanism of doxorubicin

To support the hypothesis of silanol interactions with eluting doxorubicin at low water percentage, and aiming at eliminating these possible silanol-driven contribution to retention, we have saturated the polar (silanol) sites on the stationary phase using triethylamine (TEA) [23]. For that purpose, mobile phases analogous to the ones used in the previous conditions but containing 2.54 mM l^{-1} TEA were prepared. Experiments were performed using three different buffer systems at different pH values: citrate buffer pH 2.4, acetate buffer pH 3.8 and phosphate buffer pH 5.4. Results are shown in Fig. 3.

In contrast to the results shown in Fig. 2, log k curves were linear at equivalent q values, indicating a predominant solvophobic retention mechanism. It was observed that doxorubicin retention increased when the buffer pH increased for each percentage of methanol. The concomitant increase of doxorubicin retention with buffer pH and q values is in accordance with the hypothesis of a pure reversed-phase elution mechanism described by the solvophobic theory [21]. However, the selected solvophobic elution mode compromised by a non-negligible doxorubicin decrease of retention when comparing Figs. 2 and 3. Because of the basic property of doxoruribin, one can expect an increase in retention at higher pH buffered mobile phase, as shown in Fig.

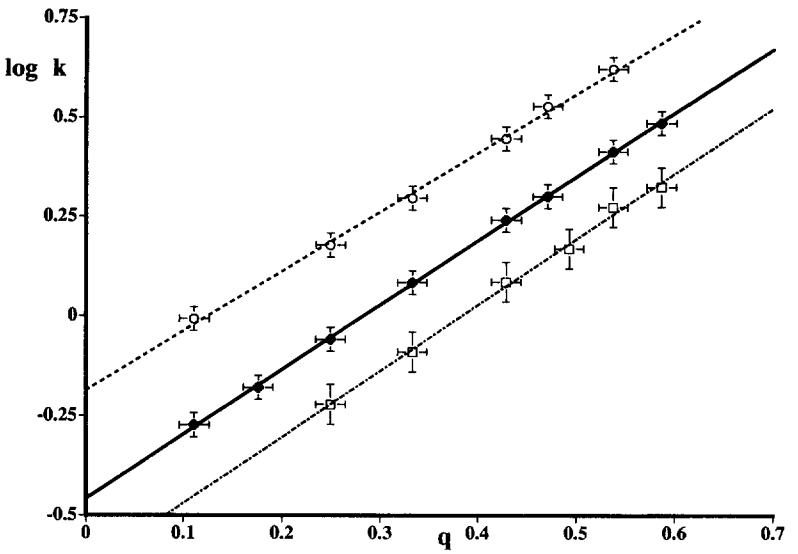

Fig. 3. Solvophobic elution mechanism of doxorubicin in the presence of triethylamine. Error bars as described in Fig. 2. For the three plotted series and considering linear-fit curves, the minimum squared residual obtained was higher than 0.95. Chomatographic conditions are described in Section 2.2. (□) Citrate buffer, pH 2.4; (♦) acetate buffer, pH 3.8; (○) acetate buffer, pH 4.5.

3, in which the percentage of ionized doxorubicin molecules will be reduced. Doxorubicin retention characteristics were tested using the Student's *t*-test. Three tests were performed (phosphate compared to acetate and citrate, acetate compared to citrate), in each case with degrees of freedom of 15 or 16, *t*-values indicated that these series were significantly different. Because it is a base, doxorubicin was more strongly retained at high pH.

Retention factors obtained under these conditions increased when the pH of the buffer increased, as predicted by the solvophobic theory applied to weak bases. However, measured retentions were short and as the procedure which consists in increasing the mobile phase pH is limited by the stationary phase stability, ion-pairing methods have been developed to overcome this limitation [24]. Hence a ion-pair reagent (heptane sulfonic acid, Sigma–Aldrich) was added to the mobile phase, taking care to maintain the mobile phase ionic strength.

3.3. Ion-pair elution mechanism of doxorubicin

To demonstrate effective ion-pair formation, experiments were performed at acidic pH using a citric acid buffer where the percentage of ionized doxorubicin was highest. Three aqueous buffers were prepared, the first one with TEA only, the second one with TEA and HX and the third one with TEA and CHX, with concentrations of HX and CHX equivalent at 4.0±0.2 m*M*.

In Fig. 4, it is shown that comparisons of the different mobile phases are possible. Retention data of doxorubicin in mobile phase with TEA can be compared to retention data in the mobile phases with TEA and ion-pair reagents added at a similar molar concentration (HX and CHX). First, when the two mobile phases containing ion-pair reagents are compared to the one containing TEA only, it can be observed that retention characteristics of doxorubicin are systematically increased. This suggests the formation of ion-pair complexes. This effect can be confirmed using statistical comparison methods of the three series of data. The methodology is identical to the one described for Fig. 3. It appears that the data obtained in the presence of the counter-ion in the mobile phase differ significantly from the one without counter-ion. However, when both counter-ions were analysed, no significant differences appeared using Student's *t*-test comparison. Moreover, as each mobile phase contained a high chloride concentration, the results shown in Fig. 4 and the statistical analysis of data allowed to conclude that even if some ion-pair formation between doxorubicin

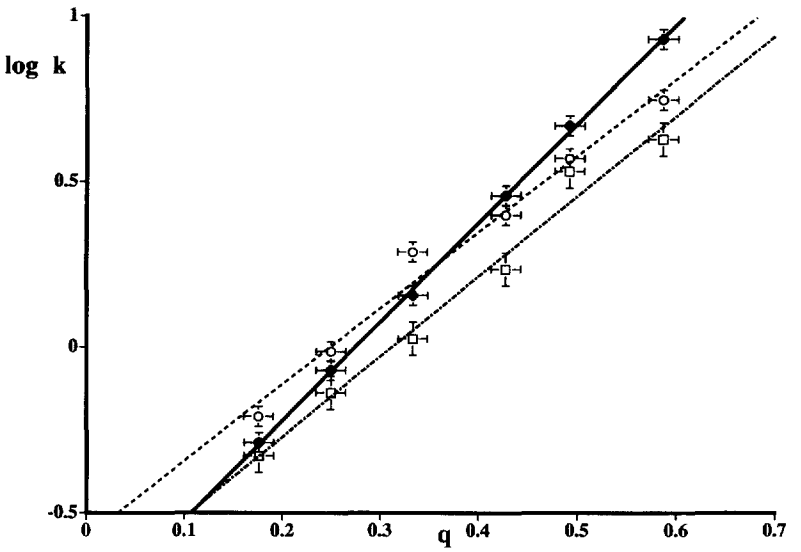

Fig. 4. Comparison of different ion-pair reagents (heptane sulfonic acid and 2-cyanohexanoic acid) at similar concentrations on doxorubicin retention factor at pH 2.2 in a citrate buffer. The minimum squared residual obtained was 0.881. Statistical procedure for series comparison and chromatographic conditions are described in Section 2.2. (□) Reversed-phase elution; (♦) heptane sulfonate ion-pair elution; (○) cyanohexanoate ion-pair elution.

and chloride was possible, these with HX and CHX were predominant.

At pH≈2.2, less than 50% of the CHX was ionized (estimated value of pK_a 2.6); nevertheless, systematic increase in retention was observed regardless of the buffer percentage in the mobile phase. Using the methodological statements defined to demonstrate the ion-pair RP-HPLC elution mechanism [25], and the previously demonstrated ion-pair RP-HPLC elution mechanism of doxorubicin [26], the results shown in Fig. 4 allowed us to state that there is a strong probability of ion-pair formation between the ionized form of doxorubicin and the ionized form of CHX, even if ion-pair formation yield is relatively low. When mobile phase containing HX and CHX where compared using the mean values of Student's *t*-test, no significant differences were found. A more detailed procedure [19] was therefore employed. Both slope and origin values of the linear regressions (log *k* versus *q*) were analysed. Linear regression analysis was performed on all series of data. Estimated variances and conditional estimated variances were calculated for each series, as well as the slope and the origin. Then, each pair of series were compared in terms of slope and origin abscissa with a *t*-test whose degree of freedom was the sum of the number of data in both series minus 4. It appeared that CHX and HX behaved differently, as well as CHX and HX compared to a mobile phase without the ion-pair reagent.

To confirm and assess the possible ion-pair association of doxorubicin with PACA compounds under conditions close to biological, a new buffer system set at pH 7.2 was prepared. For this series of experiments, ion-pair concentrations for HX and CHX mobile phase were also chosen at 3.9±0.2 m*M*. The results are shown in Fig. 5.

When retention data of doxorubicin eluted in mobile phase series made of 7.2 pH buffer with 2.54 m*M* TEA per liter and various percentages of methanol were analysed, linear fitting of log *k* as a function of *q* exhibited a correlation coefficient of 0.90 suggesting that retention of doxorubicin was prominently driven by a solvophobic mechanism [20,21]. Moreover, the systematic retention increase observed in Fig. 5 when doxorubicin is associated with CHX or HX, indicated that ion-pair formation was highly probable.

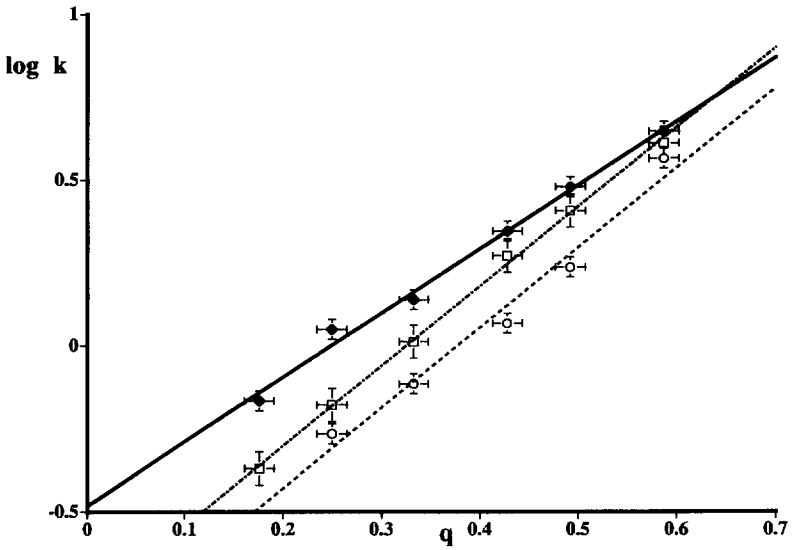

Fig. 5. Comparison of the effect of ion-pair reagents on doxorubicin retention factor at physiological pH (Tris buffer). Plotted series statistical and fitting characteristics identical to those of Fig. 3. Chromatographic conditions are described in Section 2.2. (○) Reversed-phase elution; (□) heptane sulfonate ion-pair elution; (♦) cyanohexanoate ion-pair elution.

3.4. Ion-pair elution mode of doxorubicin at physiological pH

With HX used as ion-pairing reagent, retention values of doxorubicin were generally greater as compared to retention data obtained with an analogous mobile phase without HX. In the presence of HX in the mobile phase, retention curve of log k as a function of q was linear, and at equivalent q values showed a systematic increase in retention. With the second mobile phase containing CHX (3.9 mM) retention behavior analysis of the CHX ion-pair association showed that ion-paired doxorubicin was even more retained. Therefore for each methanol percentage of the mobile phases under study it appeared that retention increased when doxorubicin was eluted with a counter-ion reagent, retention increased. Fig. 5 shows clearly that for a mobile phase buffer pH of 7.2, CHX was the most efficient counter-ion, i.e., the one which results in the highest doxorubicin retention. One can also observe that this ion-pair effect is more pronounced at high methanol percentage in the mobile phase. Even if k differences decreased at high q values, when ion-paired doxorubicin conditions are provoked, the latter compound is more strongly retained than without CHX or HX. To confirm this effect, systematic statistical comparison analyses were performed, using both statistical procedures as already described. Moreover, for q values lower than 0.35, that is for increased methanol percentage in the mobile phase, the CHX ion-pair reagent led to increased doxorubicin retention when compared to heptanesulfonic acid.

However, ion-pair formation yield of CHX with doxorubicin appeared increased with high methanol percentage in the mobile phase. Under these conditions, the dielectric constant of the mobile phase is reduced compared to more aqueous mobile phase.

4. Conclusions

Systematic studies of retention properties of doxorubicin at different pH values, in different buffer systems, with different solvent strengths, but constant ionic strength, are presented in Figs. 2–5. Results indicated, in the light of the solvophobic theory [20,21] and of ion-pair RP-HPLC mechanism [22], that doxorubicin and CHX formed an ion-pair association at pH=7.2 in aqueous mobile phase,

even if a probably low ion-pair formation yield is encountered. Therefore, the chromatographic data described in this report supports experimentally the hypothesis on the efficiency of doxorubicin loaded PACA nanoparticles [11].

With due precautions, we can imagine the chromatographic interface system as a model for cell membrane. In the chromatographic system the ion-pair formation is facilitated at the stationary-mobile phase interface where the polarity of the medium is lower than in the bulk mobile phase. By extrapolation one can expect that an analogous mechanism may appear at the interface between extracellular fluid and cell membrane facilitating, therefore, the transport into the cell.

From the biological point of view, the demonstration of ion-pair formation between doxorubicin and CHX can be extrapolated to pharmacological hypotheses with some restrictions. First, we assume that CHX and PACA compounds behave similarly in particular in terms of acidic properties. In that sense it is to be noted that CHX has been synthesised specially to be as close as possible to PACA, and that both compounds could be described as PACA-like molecules.

Secondly, we assume that experiments performed with organic and aqueous buffer phase mixtures can be extended to pure physiological aqueous medium. Arguments shown in Fig. 5 support this second hypothesis, as ion-pair formation are still observed at high q values (aqueous mobile phase).

Therefore, under physiological conditions, as doxorubicin exhibits a pK_a value of 8.4, at physiological pH (7.4) this weak base is mainly in its ionized form. When just diluted in the cell suspension and nutritive medium, its diffusional rate across cell membrane is unfavored because of repulsive electrostatic interactions between charged amino groups of the anthracyclin and the charged groups of membrane phospholipids [27]. Masking the charged amino group of antracyclin (negatively charged) by the carboxylic groups of CHX by means of an ion-pair formation will therefore decrease the global charge of the drug and will increase its hydrophobicity by addition of the apolar chains of the counter-ion. The supramolecular structure therefore formed appears to be globally more hydrophobic and less charged, facilitating the diffusional intake of the drug by the cell. This is made possible, for example, by the presence of PACA (PACA compound of acidic characteristics) near the cell membrane, the which will act as counter-ions as well as CHX acted in the chromatographic system.

Under biological conditions, when doxorubicin loaded PACA nanoparticles are incubated with cells in culture, they are supposed to adsorb onto the cell surface [9]. Because of the hydrolysis process of the nanoparticles, doxorubicin and PACA compounds are progressively released directly in the vicinity of the cells membrane. The rate of doxorubicin release from these nanoparticles has been shown to be an important parameter conditioning the amount of doxorubicin taken up by the resistant cells [28]. This can be related to a lower transmembrane potential in the case of resistant cells compared to the sensitive parent line [29]. Such a lower potential inhibits the diffusion of cationic drugs to a large extent [30], and is responsible for drug resistance. Charge masking by the mean of an ion-pair will therefore restore drug accumulation by restoring a convenient drug diffusion rate. In terms of ion-pair stability, the low dielectric constant of the cellular membranes would favor the formation of ion-pairs [31]. However, if such a doxorubicin–PACA complex exists, an excessive affinity of the ion-pair for the lipidic membrane, would lead to drug accumulation inside the cell membrane. Even if this happens, it would probably not inhibit doxorubicin cytotoxicity, since doxorubicin behaved toxic effects in the nucleus and in the membrane [32]. Finally, if ion-pairs are present, it would involve the sugar moiety of doxorubicin molecules. An example in the literature shows that grafting hydrophobic groups onto the amine function of doxorubicin does not inhibit drug cytotoxicity, and even increases the antitumor efficiency in the case of drug resistance [33]. The hypothetic ion-pair association whose representation is described in Fig. 1 is thus not a limiting factor for drug cytotoxicity.

The average polymerization number of PACA compounds when hydrolysed from nanoparticles is 3 to 4, indicating that each liberated soluble polycyanoacrylic acid may carry 3 to 4 carboxylic groups. In terms of ion-pair formation, association between one polycyanoacrylic acid and up to 3 to 4 doxorubicin molecules can be imagined. It can be argued that steric inhibition may hinder the formation of that complex, but doxorubicin molecules are known to stack [1] leading to a minimal steric

volume. Although the exact structure cannot be to date determined, FTIR and RAman spectroscopy have already assessed the presence of both doxorubicin and polycyanoacrylic acid in a precipitate obtained by mixing doxorubicin and bioeroded PACA nanoparticle solutions [28], which again, supports the hypothesis of the ion-pair formation.

The general methodology used in this study to characterize the potential of a cyanoacrylic acid to form an ion-pair with doxorubicin can be applied to other anionic molecules. Screening candidates for doxorubicin encapsulation is then simplified, i.e., only compounds able to give ion-pairs with doxorubicin should be qualified at first for further investigation on tumor resistant cell lines.

Acknowledgements

The Board of the Analytical Chemistry Departement of the College of Pharmacy, Université Paris-Sud, is thanked for organisation assistance as this research project was integrated into an undergraduate analytical chemistry course. J.M. Marbouty, Head of English for Specific Purpose Department of College of Pharmacy, Limoges University is also acknowledged for English language corrections.

References

[1] S.T. Crooke and S.D. Reich (Editors), Anthracyclines, Academic Press, New York, 1980.
[2] J. Kattan, J.P. Droz, P. Couvreur, J.P. Marino, A. Boutan-Laroze, P. Rougier, P. Brault, H. Vranckx, J.M. Grognet, X. Morge, H. Sancho-Garnier, Invest. New Drugs 10 (1992) 191.
[3] N. Chiannilkulchai, Z. Drouich, J.P. Benoit, A.L. Parodi, P. Couvreur, Select. Cancer Therap. 5 (1989) 1.
[4] C. Cuvier, L. Roblot-Treupel, J.M. Millot, G. Lizard, S. Chevillard, M. Mnafait, P. Couvreur, M.F. Poupon, Biochem. Pharmacol. 44 (1992) 55.
[5] F. Nemati, C. Dubernet, A. Colin De Verdiere, M.F. Poupon, L. Treupel-Acar, F. Puisieux, P. Couvreur, Int. J. Pharmaceutics 102 (1994) 55.
[6] S. Bennis, C. Chapey, P. Couvreur, J. Robert, Eur. J. Cancer 30A (1993) 106.
[7] I.B. Robinson, Clin. Physiol. Biochem. 5 (1987) 140.
[8] V. Cuise, P. Jaffray, J. Delattre, F. Puisieux, M. Adolphe, P. Couvreur, Cell. Mol. Pharmacol. 33 (1987) 397.
[9] A. Colin De Verdiere, C. Dubernet, F. Nemati, M.F. Poupon, F. Puisieux, P. Couvreur, Cancer Chemother. Pharmacol. 33 (1994) 504.
[10] V. Leanerts, P. Couvreur, D. Cristens-Leyh, E. Joiris, M. Roland, B. Rollman, P. Speiser, Biomaterials 5 (1984) 65.
[11] C. Dubernet, F. Nemati, A. Colin De Verdiere, M.F. Poupon, F. Puisieux and P. Couvreur, Proc. 1st World Meeting APGI/APV, Budapest, May 9–11 1995. p. 473.
[12] C. Dubernet, A. Colin De Verdiere, F. Nemati, M.F. Poupon, F. Puisieux, P. Couvreur, Proc. Int. Symp. Control Rel. Bioact. Mater. 21 (1994) 202.
[13] F. Nemati, C. Dubernet, H. Fessi, A. Colin de Verdiere, F. Puisieux, P. Couvreur, Int. J. Pharmaceutics 138 (1996) 237–246.
[14] W. Priebe, N.T. Van, T.G. Burke, R. Perez-Soler, Anti-Cancer Drugs 4 (1993) 37.
[15] M.A. Van Lancker, H.J.C.F. Nelis, A.P. Leenheer, J. Chromatogr. 254 (1983) 45.
[16] L. Vansnick, P. Couvreur, D. Chistiaens-Leyh, M. Roland, Pharm. Res. 1 (1985) 36.
[17] D.D. Perrin, B. Dempsey and E.P. Serjeant, pK_a Determination for Organic Acids and Bases, Chapman and Hall, London, 1974.
[18] B. Tremillon, Electrochimie Analytique et Reaction en Solutions, Vol. 1, Masson, Paris, 2nd ed., 1993, Ch. VI, p. 256.
[19] D.G. Kleibaum and L.L. Kupper, Applied Regression Analysis and other Multivariate Methods, Dubury Press, London, 1978, p. 98.
[20] E. Tomlinson, T.M. Jefferies, C.M. Riley, J. Chromatogr. 159 (1978) 315.
[21] C. Horwäth, W. Melander, I. Molnär, J. Chromatogr. 125 (1976) 129.
[22] D.S. Lu, J. Vialle, H. Tralongo, R. Longeray, J. Chromatogr. 268 (1983) 1.
[23] L.R. Snyder, J.L. Glajch and J.J. Kirkland, Practical HPLC Method Development, Wiley Interscience, 1988, Ch. 3, p. 57.
[24] K.G. Wahlund, J. Chromatogr. 115 (1975) 411.
[25] J.C. Kraak, K.M. Jonker, J.F.K. Huber, J. Chromatogr. 142 (1977) 671.
[26] G. De Groot, B.C.A. Tepas, G. Storm, Anal. Chim. Acta 217 (1989) 149.
[27] C.A. Manella, Q. Wang, Biochim. Biophys. Acta 981 (1989) 363.
[28] A. Colin de Verdière, C. Dubernet, F. Nemati, E. Soma, M. Apple, J. Feré, S. Bernard, F. Puisieux and P. Couvreur, Br. J. Cancer, (1997) in press.
[29] S. Gupta, B. Vayuvegula, P. Sweet, M. Stepeckey, S. Murray, R. Jacobs, L. Slater, Clin. Res. 34 (1986) 881A.
[30] A. Ramu, N. Ramu, R. Gorodetsky, Biochem. Pharmacol. 42 (1991) 1699.
[31] R.B. Gunn, in T.E. Andreoli, J. Hoffman and D.D. Fanesil (Editors), Physiological Membrane Disorders, Plenum Press, New York, 1978, p. 243.
[32] T.R. Tritton, G. Yee, L.B. Wingard, Fed. Proc. 42 (1983) 284.
[33] J. Nafziger, G. Averland, E. Bertoumesque, G. Caudel, C. Monneret, J. Antibiot. (Tokyo) 48 (1995) 1185.