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Bioanalysis of some anthracyclines in human plasma by capillary electrophoresis with laser-induced fluorescence detection

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ABSTRACT

A rapid method for the determination of daunorubicin, doxorubicin and epirubicin in human plasma is described. Samples are pretreated and concentrated by liquid–liquid extraction with chloroform and back-extraction into phosphoric acid, respectively. This pretreatment results in a sample matrix of low ionic strength in comparison with the electrophoresis buffer, permitting a 20–30-fold increase in the injected amount by zone sharpening when electrokinetic injection is applied. Analyte interaction with the capillary wall is prevented by using high acetonitrile contents in the electrophoresis buffer, which results in reproducible migration times and highly efficient separations. Laser-induced fluorescence detection provides an extremely sensitive and selective method without detectable biological interferences. The limit of determination of daunorubicin, epirubicin and doxorubicin in plasma ranges from 125 to 250 pg/ml.

INTRODUCTION

The drug doxorubicin (DOX) and its epimer epirubicin (EPI) are two of the most frequently used anthracycline antibiotics in the treatment of a wide variety of cancers. Because of the serious side-effects of chemotherapy, there is a continuous search for analogues with better therapeutic characteristics. As a result, numerous analytical procedures have been developed for anthracyclines and several reviews have been published. The analytical aspects of cytostatic drug analysis have recently been reviewed [1]. High-performance liquid chromatography (HPLC) is frequently used for the determination of anthracyclines and, especially in combination with fluorescence detection, low detection limits can be achieved. Under acidic conditions the anthracyclines show native fluorescence [1–3].

Capillary electrophoresis (CE) is a separation technique that is characterized by extremely high efficiencies and short analysis times [4,5], recently reviewed by Kuhr [6]. However, although plate numbers of more than 10^6 have been reported, these efficiencies are not always obtained in bioanalytical applications. A biological matrix, especially plasma, contains high concentrations of interfering compounds such as proteins and salts. Therefore, a sample pretreatment resulting in a matrix that is compatible with the CE buffer (*i.e.*, deproteinated, same ionic strength) is of extreme importance for reproducible analysis.

The injection volumes in CE are of the order of 0.1–10 nl. This implies that in principle only a few microlitres of sample have to be pretreated, of which only a fraction will be analysed. In this assay the sample volume to be pretreated is only limited by the amount of analyte that can be handled without decreases in reproducibility and sensitivity due to aspecific adsorption on the surface of sample vials and other laboratory materials.

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As a consequence of the use of narrow-bore capillaries with a small optical path length, the concentration detection limits in CE are relatively high. Highly sensitive detectors such as systems based on laser-induced fluorescence (LIF) are essential and several publications have reported very low detection limits [7].

The literature on the application of CE in the bioanalysis of drugs in plasma samples is limited, demonstrating the difficulties of working with biological matrices. Roach *et al.* [8] presented a method for the biodetermination of methotrexate in plasma using solid-phase isolation, followed by a concentration step before CE analysis. Recently, Nishi *et al.* [9] described the determination of aspoxicillin in plasma with direct sample injection using micellar electrokinetic capillary chromatography (MECC). Sodium dodecyl sulphate (SDS) in the electrophoresis buffer complexes with the plasma proteins and prevents protein adsorption on the capillary wall.

In this paper we describe the biodetermination of some anthracyclines using CE in combination with LIF. For the optimization of the CE analysis a test mixture of DOX and EPI is used, which are stereoisomers differing only in that the hydroxy group at C-4′ of the daunosamine is inverted. For calibration graphs for both drugs daunorubicin (DAU) was used as an internal standard, differing from DOX in that an extra hydroxyl group is present on the adriamycinone [1,3]. The applicability of the method is demonstrated with the analysis of samples from cancer patients treated with EPI.

EXPERIMENTAL

Equipment

CE was carried out in a 700 × 0.075 mm I.D. fused-silica capillary (SGE, Ringwood, Victoria, Australia) using an electrophoresis buffer composed of acetonitrile (ACN) in 100 m*M*, pH 4.2 sodium phosphate buffer (PB) (7:3, v/v). The voltage (20–25 kV) was supplied by a Model RR100-1.5P power supply (Gamma High Voltage Research, Mt. Vernon, NY, USA) operating in the constant-current mode at 35 μA. The current was measured over a 350-Ω resistance in series between the cathode and earth using a microamperometer (Model 134312; Goerz, Vienna, Switzerland). Samples were injected electrokinetically, applying 12 kV for 5 s.

On-capillary LIF detection took place at 650 mm from the anodic end, using a water-cooled argon ion laser (Model 2025-03; Spectra-Physics, Mt. View, CA, USA) lasing at 476.5 nm at 80 mW for excitation. For comparison of detection limits an air-cooled argon ion laser (Model 162D07; Spectra-Physics) operating at 5 mW at 488 nm was also used.

The laser beam was focused on the capillary from which the polyimide coating over a length of several millimetres had been removed. Fluorescent radiation was transported to the photomultiplier tube (PMT) through a liquid light guide (1000 × 5.0 mm I.D.) (Model 77556; Oriel, Stratford, CT, USA) equipped with a planoconvex fused-silica lens (*D* 11 mm, focal length 19 mm) (Model 41210) at each end. The fluorescent radiation was directed on to a 595-nm band pass interference filter (10 nm band width, Type 53920; Oriel). The PMT (Model RFIB235F; Thorn EMI, Ruislip, Middlesex, UK) was operated at 800 V (power supply Model PM28B; Thorn EMI).

The signal was amplified by a current amplifier (Model 427; Keithley Instruments, Cleveland, OH, USA) and digitized using a laboratory-made 12-bit A/D converter operating at a frequency of 20 Hz. The A/D converter was connected to a computer (Atari Mega ST4; Atari, Sunnyvale, CA, USA) which controlled the operating voltage, the injection voltage, the injection time, the sampling frequency of the converter and the data handling.

Chemicals

Doxorubicin (Adriblastina) and epirubicin (Farmorubicine) were both available as 1 mg/ml hydrochloride solutions containing 5 mg ml^{-1} of lactose and were purchased from Carlo Erba (Milan, Italy). Daunorubicin (Cerubidine) was purchased from Rhône-Poulenc (Paris, France). Chloroform (J. T. Baker, Deventer, Netherlands) and 85% phosphoric acid (Merck, Darmstadt, Germany) were of analytical-reagent grade. Acetonitrile (Rathburn, Walkerburn, UK) was of HPLC grade and degassed and sucked through a

0.2-μm membrane filter (Type SM 11606; Sartorius, Breukelen, Netherlands) after mixing with 100 mM sodium dihydrogenphosphate (Merck) buffer. Demineralized water was used throughout.

Calibration graphs and extraction

Plasma sample were spiked with EPI and DOX solution at a concentration in the range of 0.3–300 ng ml^{-1} by adding 100 μl of EPI and DOX (in 10 mM, pH 4.2 PB containing 10% of ACN) to 900 μl of plasma containing 111 or 5.5 ng ml^{-1} of DAU in 10-ml polyethylene vials. A final DAU concentration of 100 ng ml^{-1} was used as internal standard (IS) for analysis of patients' samples. For assay validation 5 ng ml^{-1} DAU was used. A 1-ml volume of spiked plasma was extracted by adding 2 ml of chloroform, vortex mixing for 1 min and centrifuging for 10 min at 1000 g. A volume of 1.6 ml of the lower organic layer was removed, extracted with 100 μl of 5 mM, pH 2.3 phosphoric acid, vortex mixed and centrifuged in the same way. A 50-μl volume of the upper layer was removed and used for CE analysis after addition of 150 μl of ACN, resulting in a final ACN concentration of 75%. Patients' samples were treated in the same way except that, instead of adding EPI or DOX, 100 μl of 1.0 μg/ml DAU were added to 900 μl of serum.

RESULTS AND DISCUSSION

Development of the CE system

In preliminary experiments with a phosphate buffer, DAU, DOX and EPI were not separated. Under such conditions, poor performance arose owing to interactions with the capillary wall (Fig. 1a). By modifying the electrophoresis buffer with 70% ACN, the interaction of the analyte with the capillary wall decreased and the peak shape and the resolution of DAU, DOX and EPI was improved dramatically (Fig. 1b). Further, the addition of ACN to the electrophoresis buffer caused a decrease in the conductivity and consequently resulted in reduced heat generation inside the capillary. When the same sodium phosphate concentration was diluted (3:7) with water instead of ACN, the measured current was three times higher (105 *vs.* 35 μA). Owing to the resulting decrease in the radial temperature gradient over the

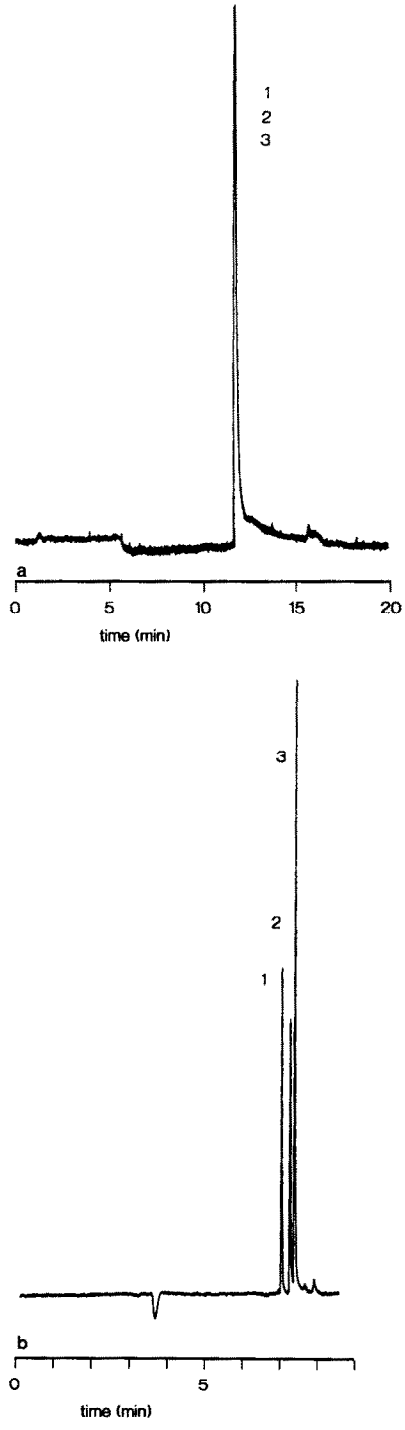

Fig. 1. Effect of the addition of acetonitrile to the electrophoresis buffer on the separation of anthracyclines. (a) CE analysis of a mixture of 1 μg ml^{-1} of (1) DAU, (2) EPI and (3) DOX in 20 mM PB (pH 4.2) using a voltage of 20 kV over a 70 cm × 75 μm I.D. capillary (65 length cm to detection). The injection was made electrokinetically at 10 kV for 5 s. (b) Separation of 100 ng ml^{-1} of (1) DAU, (2) EPI and (3) DOX in 100 mM PB (pH 4.2) diluted 3:7 with ACN. The injection was made electrokinetically at 12 kV for 5 s; other conditions as in (a).

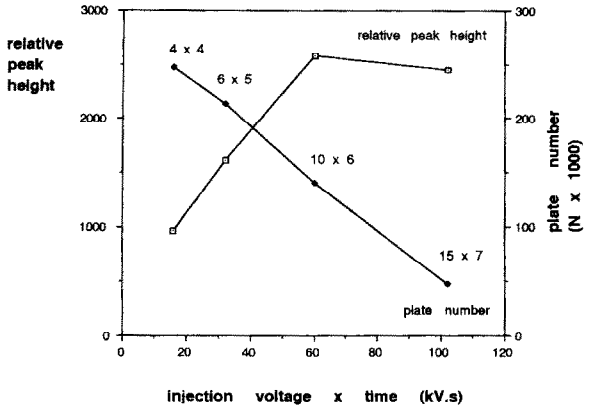

Fig. 2. Effect of injection volume on the efficiency and the peak height for a 100 ng ml^{-1} solution of DOX in electrophoresis buffer. CE conditions as in Fig. 1b.

capillary with the ACN-containing buffer, the efficiency was improved. Similar effects have been reported for the separation of positional isomers of substituted benzoic acids [10].

The field strength was optimized at 33 kV m^{-1}. Higher field strengths resulted in decreases in efficiency and resolution because of the increased heat generation in the capillary. Early reports on CE described a linear relationship between the efficiency and the applied voltage when diffusion is the major contributor to peak broadening [5]. However, when capillaries with diameters above 50 μm are used, the Joule heat becomes an important source of peak broadening and an optimum applied voltage exists with respect to efficiency [11].

In the present assay, relative peak heights and areas were calculated using an internal standard for quantification, implying that it is not necessary to know the exact sample volume injected. Further, it is not necessary to make corrections for the discrimination of analytes with different mobilities. Discrimination in principle occurs when electrokinetic injection is applied [12]. However, for optimization purposes it is interesting to consider the injection volume. In terms of detectability high injection volumes are favourable, but the resolution decreases as a result of the broader zones (Fig. 2). Some workers have described the maximum loadability of the capillary without a substantial decrease in efficiency to be 1% of the total capillary volume [11]. The injection volume in electrokinetic injection for a certain compound is determined by both the injection voltage and the duration of the pulse. Therefore, we characterized the injection volume as a block (voltage × duration). The injection block was increased from 4 kV × 4 s to 15 kV × 7 s. Injection blocks larger than 12 kV × 5 s resulted in broader peaks, not in a higher fluorescence signal (Fig. 2). An injection at 12 kV for 5 s was chosen, corresponding to a plug length of 3.2 mm and a plug volume of 14 nl, being 0.5% of the total volume and resulting in plate numbers of *ca.* 170 000 for DOX. The injection volume and plug length were calculated from the total electrophoretic mobility of DOX ($3.24 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1}). This calculation is only relevant when the mobility in the sample matrix is the same as that in the electrophoresis buffer, *i.e.*, same pH, ionic strength, viscosity, etc. Therefore, the optimization was done with a mixture of 100 ng ml^{-1} DOX and 500 ng ml^{-1} EPI in electrophoresis buffer. This also implies that no zone sharpening took place.

Laser-induced fluorescence detection

The influence of the laser power of the water-cooled argon ion laser on the signal and noise level for DOX was investigated. An optimum appeared in the minimum detectable concentration (MDC), calculated as the concentration which results in a signal-to-noise ratio of 3, at 100 mW (Fig. 3a). Owing to the photodegradation of DOX, the fluorescence signal is not proportional to the laser power, in contrast with the noise resulting in an increased MDC at higher laser powers.

As the excitation maximum of anthracyclines is between 450 and 520 nm and the laser can supply several lasing wavelengths between 457 and 515 nm, the MDC at different lasing lines was determined. The fluorescence signal (peak height) of a 500 ng ml^{-1} DOX solution in electrophoresis buffer after injection at 20 kV for 10 s and the noise level were measured. The MDC for DOX was 1.24, 0.75, 1.09 and 1.39 ng ml^{-1} for excitation at 457.9, 476.5, 488.0 and 514.5 nm, respectively. Excitation at 476.5 nm resulted in a higher fluorescence signal and a lower noise in comparison with the other wavelengths (Fig. 3b).

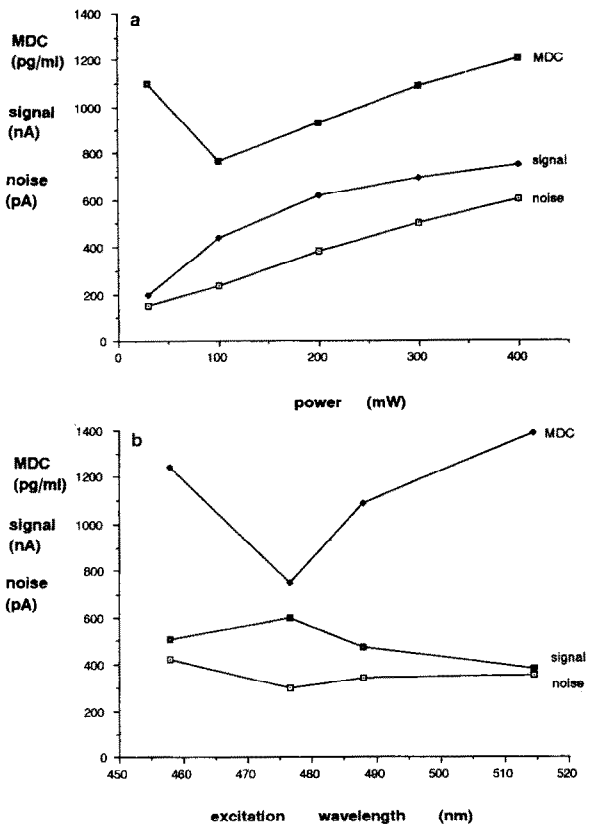

Fig. 3. Effect of (a) the power at 488 nm and (b) the wavelength at 100 mW of the excitation laser beam on the fluorescence of a 500 ng ml^{-1} DOX solution in electrophoresis buffer and the noise level. See text for other conditions.

The DOX was dissolved in electrophoresis buffer, which means that no preconcentration by stacking during injection took place. The injection was done by overloading the capillary in order to obtain a maximum and reproducible fluorescence signal. As can be seen in Fig. 2, injection blocks larger than 80 kV s give a maximum fluorescence and variations in injection volume do not affect peak heights, only the peak area.

Experiments with an inexpensive air-cooled argon ion laser operating at 5 mW and 488 nm showed an MDC for DOX in electrophoresis buffer (*i.e.*, without zone sharpening) of 8 ng ml^{-1}. Although a factor of nine higher in detection limit, this type of laser should be usable for the described anthracycline assay, considering a 20–30-fold increase in detectability due to the pretreatment and zone sharpening (see below).

Sample treatment

DOX and its analogues are known to adsorb on glassware and laboratory materials [1], introducing a source or irreproducibility, especially at low concentrations. Therefore, care was taken that all anthracycline solutions contained at least 10% ACN. To demonstrate the necessity for ACN, a dilution was made of 100 ng ml^{-1} EPI in 10 m*M* phosphate buffer (pH 4.2) in a 10-ml polyethylene vial. The fluorescence signal was measured and considered to be 100%. An aliquot was transferred from the 10-ml vial to a 1.5-ml polyethylene Eppendorf vial and the fluorescence was immediately measured. The fluorescence signal was reduced to 30% and after vortex mixing the 1.5-ml vial for 5 s the signal was reduced to 15%. However, when the same experiment was done in the presence of 10% of ACN, no decrease in fluorescence was observed.

Determination in plasma

In preliminary experiments for the biodetermination of anthracyclines, a sample pretreatment consisting of a deproteination step of spiked plasma samples by addition of 400 μl of ACN to 200 μl of plasma (66% ACN) resulted in a rapid and reproducible method. The deproteinated plasma samples were analysed directly without further pretreatment and no interferences from the plasma matrix could be observed. However, because of the diluting sample pretreatment, the detection limit of DOX in plasma was 30 ng ml^{-1}, which is

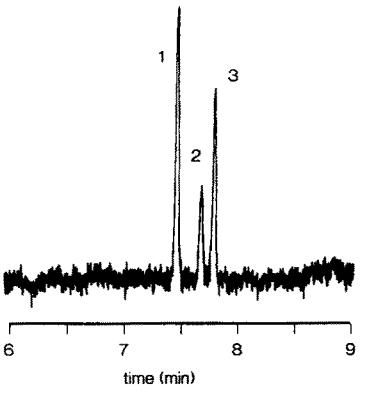

Fig. 4. Electropherogram of an extract of blank plasma spiked with (1) 1 ng ml^{-1} DAU, (2) 500 pg ml^{-1} of EPI and (3) 500 pg ml^{-1} of DOX. CE conditions as in Fig. 1b. See text for sample pretreatment procedure.

not low enough for analysis of plasma samples from cancer patients. Therefore, a concentrating sample pretreatment using liquid–liquid extraction and back-extraction was applied.

The MDCs of DAU, EPI and DOX in plasma based on a signal-to-noise ratio of 3 are 50, 70 and 35 pg ml^{-1}, respectively (Fig. 4). The limits of determination, defined as the concentration of analyte in plasma resulting in a signal-to-noise ratio of 10, are 175, 250 and 125 pg ml^{-1}, respectively. The double liquid–liquid extraction which results in a sample matrix with a low ionic strength (5 m*M* phosphoric acid–75% ACN) appears to be very advantagous regarding the detection limits because of a strong zone-sharpening effect during electrokinetic injection. This zone sharpening results in a 20–30-fold increase in peak height. Also, the efficiencies of the anthracyclines improved to plate numbers ranging from 300 000 to 500 000.

The peak concentrating effect was measured for a mixture of DAU, DOX and EPI with a concentration of 100 ng ml^{-1} after dilution. When the dilution was made in electrophoresis buffer the signals were 20–30 times lower compared with a dilution in 5 m*M* phosphoric acid to which 75% ACN was added. The MDCs for DAU, EPI and DOX in electrophoresis buffer without zone sharpening are 1.4, 1.5 and 0.9 ng ml^{-1}, respectively. An overall improvement in detectability by a factor 20–30 with respect to the MDC in plasma is realized by zone sharpening (a factor of 20–30), a concentrating pretreatment (a factor of 8), the recovery (a factor of 0.4–0.7, see validation below) and a dilution (a factor of 0.25) because of the addition of 75% ACN to the extraction buffer before CE analysis. An additional advantage of the extraction procedure in combination with the high ACN percentage in the electrophoresis buffer is that there is no need for cleaning steps between runs. The extraction procedure results in a clean and well defined sample matrix, while the high ACN content prevents interaction of the analytes with the capillary wall. This resulted in a relative standard deviation in migration times smaller than 0.5% ($n = 5$), which corresponds to a standard deviation of 2 s at a migration time of *ca.* 8 min for the three anthracyclines.

Validation

A typical calibration graph for EPI is plasma, used for analysis of patients' samples, corresponded to the calculated regression equation $y = (8.66 \pm 0.08) \cdot 10^{-3} x + (0.017 \pm 0.017)$ ($r = 0.99997$), where y is the ratio of the peak areas of EPI to DAU, x is the concentration of EPI (ng ml^{-1}) and r is the correlation coefficient.

The precision of the assay was measured as the inter-day and intra-day variability for EPI and

TABLE I

VALIDATION OF THE ANTHRACYCLINE ASSAY

Mean values and C.V. values are given for $n = 4$ in all instances, except for the inter-day variation, where $n = 3$.

Concentration (ng ml^{-1})	Epirubicin				Doxorubicin			
	Mean (ng ml^{-1})	C.V. (%)	Inaccuracy		Mean (ng ml^{-1})	C.V. (%)	Inaccuracy	
			ng ml^{-1}	%			ng ml^{-1}	%
Inter-day variability								
0.5	0.493	1.7	0.007	1.4	0.509	3.5	0.009	1.8
5	5.074	5.4	−0.074	−1.5	4.962	1.9	−0.038	−0.8
10	10.127	2.0	−0.127	−1.3	10.224	2.4	0.224	2.2
Intra-day variability								
0.5	0.486	3.0	0.014	2.9	0.501	6.9	0.001	0.2
5	4.907	1.0	0.093	1.9	4.897	1.9	−0.103	−2.1
10	10.194	3.3	0.194	1.9	10.210	2.0	0.210	2.1

DOX in plasma (n = 4) at concentrations of 0.5, 5 and 10 ng ml^{-1}. The intra-day precision was calculated as the coefficient of variation (C.V.) of the four determinations and varied from 1.0 to 6.9% (Table I). The inter-day precision was calculated as the C.V. of the mean of the measured concentrations on three different days (n = 3) and varied from 1.7 to 5.4% (Table I). The inaccuracy of the assay was determined by calculating the difference between the measured and the real concentration (Table I).

The reproducibility of the CE system only was determined for six injections of a 1 μg ml^{-1} solution of DAU and DOX in electrophoresis buffer. The C.V. of the peak areas was 7% for DOX and 8% for DAU and that of the peak-area ratios was 2.5%.

The recovery was measured for different concentrations of EPI and DOX at 0.5, 5 and 50 ng ml^{-1} in plasma (n = 4). A correction in the calculation was made for the 20% loss of chloroform where 1.6 ml of the total of 2.0 ml was re-extracted and for a concentration factor of 10 (1 ml of plasma to 100 μl of phosphoric acid). This results in a theoretical concentration factor of 8. Concentration factors of 3.5–5.6 corresponding to recoveries of 45–70% with C.V. values of 16–8% were measured (Table II).

The usefulness of the developed method was demonstrated with the analysis of plasma samples from a cancer patient treated with 50 mg m^{-2} of EPI. The CE analysis of the plasma samples shows a remarkably clean background, which is caused by the combination of the extraction procedure and the selective detection (Fig. 5a). There are not many compounds in the biological matrix having native fluorescence in the visible part of the spectrum. Fig. 5b shows the plasma concentration time course for the same patient.

TABLE II

RECOVERIES OF DOXORUBICIN AND EPIRUBICIN

The mean values of four determinations are given for each concentration.

Concentration (ng ml^{-1})	Recovery (mean ± C.V.) (%)	
	EPI	DOX
0.5	55 ± 16	50 ± 15
5.0	55 ± 8	45 ± 8
50.0	70 ± 8	66 ± 8

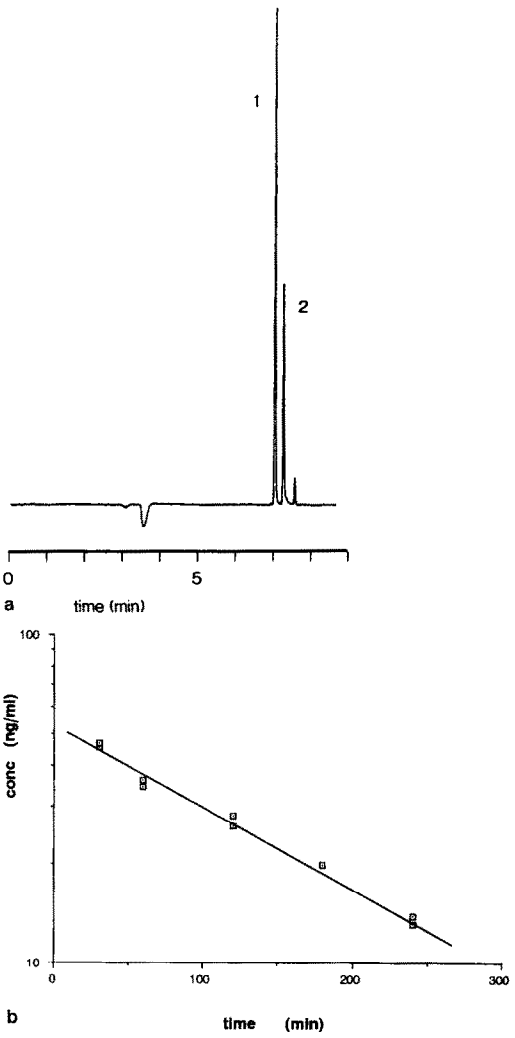

Fig. 5. (a) Electropherogram of an extract of a plasma sample taken 120 min after administration from a patient treated intravenously with 50 mg m^{-2} of EPI (2) intravenously; 100 ng ml^{-1} of DAU (1) was used as internal standard. The EPI peak corresponds to 27 ng ml^{-1}. The small third peak could not be identified. (b) Plasma concentration time course from the same patient.

CONCLUSION

A sensitive and selective assay for the determination of anthracyclines in plasma has been pre-

sented. Detection limits in the pg ml^{-1} range have been obtained, resulting in an interesting alternative for high-performance liquid chromatographic methods where analyses may be disturbed by interferences originating from the biological matrix or where the selectivity and efficiency are not satisfactory.

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