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High-performance liquid chromatography of anthracycline antibiotics with electrochemical detection. Application to the clinical pharmacokinetics of 4′-deoxydoxorubicin

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Abstract: The high-performance liquid chromatography with electrochemical detection (HPLC-EC) of six anthracycline antibiotics is described. All the compounds of interest may be analysed using a reversed-phase column (ODS Hypersil or Zorbax ODS) and a mobile phase consisting of acetonitrile–isopropanol–0.1 M phosphate buffer (pH 4.5). The use of short columns (7.5 cm) allowed short analysis times and improved sensitivity, such that detection limits of between 1 and 2 ng ml^{-1} could be achieved. Sample preparation involved an alkaline extraction into chloroform, followed by a back extraction into acid solution. Final clean-up of the samples is achieved by shaking the final acidic extract with an organic solvent. The precision of the assay for the compounds studied ranges between 1.22 and 6.46% and the accuracy ranges between 94.5 and 106.0%. The analytical methodology developed is applied to the clinical pharmacokinetics of 4′-deoxydoxorubicin. The correlation between plasma levels found by HPLC-EC and those found by HPLC with fluorescence detection is excellent (r^2 = 0.990).

Keywords: *High-performance liquid chromatography; electrochemical detection; anthracycline antibiotics; clinical pharmacokinetics.*

Introduction

Daunorubicin (DNR) and doxorubicin (DOX) are the most widely used of the anthracycline antibiotics [1]. They play a major role in the effective treatment of a wide variety of cancers including Hodgkin's disease, non-Hodgkin's lymphomas and acute leukaemia [1]. Due to their serious acute and chronic toxicities, the worst being cardiac toxicity [2], there is a continuous search for analogues of DNR and DOX which show greater potencies coupled with reduced side effects. Some of the analogues (Fig. 1) which appear to have therapeutic indices better than DNR and DOX include 4′-epidoxorubicin (EPI), 4′-deoxydoxorubicin (DEOXY), 3′-deamino-3′-(3-cyano-4-morpholino)doxorubicin (CN) and 4-demethoxydaunorubicin (DMDR).

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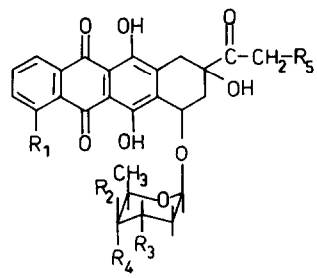


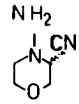
R_1	R_2	R_3	R_4	R_5	
CH_3O	H	NH_2	OH	OH	DOX
CH_3O	OH	NH_2	H	OH	EPI
H	H	NH_2	OH	H	DMDR
CH_3O	H	NH_2	H	OH	DEOXY
CH_3O	H		OH	OH	CN
CH_3O	H	NH_2	OH	H	DNR

Figure 1
The structures of doxorubicin (DOX), 4′-epidoxorubicin (EPI), 4-demethoxydaunorubicin (DMDR), 4′-deoxydoxorubicin (DEOXY), 3′-deamino-3′-(3-cyanomopholino)doxorubicin (CN) and daunorubicin (DNR).

Epimerization of the hydroxyl group in the 4′ position of the amino sugar gives EPI which has been shown to be less cardiotoxic and more extensively metabolized than DOX in rats [3]. Removal of the 4′-hydroxy group gives DEOXY which has greater potency and less cardiac toxicity than DOX in a mouse model [3]. One of the most potent anthracycline antibiotics synthesized so far is CN which has been found to be 600 times more potent than DOX against P388 leukemia in mice [4]. In addition, it has been reported that CN does not produce myocardial lesions following chronic administration of the drug to mice [5]. CN exists as a pair of diastereomers due to the lack of chiral control in the attachment of the cyano group to the morpholine ring during synthesis. The demethoxy analogue of daunorubicin, DMDR, has about eight times the antineoplastic activity of its parent compound; however, its toxicity is also increased [3]. These compounds are undergoing extensive clinical investigations. As a prerequisite of pharmacokinetic investigations sensitive analytical procedures are required for their determination in biological fluids.

High-performance liquid chromatography (HPLC) has been used extensively [e.g. refs 6–10] for the analysis of anthracycline antibiotics in plasma. Almost exclusively, HPLC methods for the analysis of this group of drugs employ fluorescence detection which give limits of detection of around 5 ng ml^{-1} in plasma. In contrast with fluorescence detectors, the use of electrochemical detectors in combination with HPLC for the analysis of anthracycline antibiotics has received much less attention [11, 12]. This is despite the fact that these compounds contain both oxidizable (phenolic) and reducible (quinone) functional groups and are ideally suited to electrochemical detection. The high-performance liquid chromatography with electrochemical detection (HPLC-EC) of DNR and DOX has been described by Akpofure *et al.* [11] and Kotake *et al.* [12],

respectively. The limits of detection were reported to be 8 ng ml^{-1} for DNR [11] and 2 ng ml^{-1} for DOX [12]. Present studies are concerned with investigating the potential of HPLC-EC for the analysis of anthracyclines as a class of compounds using DOX, DNR and four of their analogues as representative examples. Following the development of the analytical methodology, the techniques have been applied to a variety of *in vitro* and *in vivo* studies. In this study the application of HPLC-EC to the pharmacokinetics of DEOXY is demonstrated.

Experimental

Chemicals and reagents

The doxorubicin was a gift from Adria Laboratories, Columbus, OH. The analogues of DOX and DNR were kindly provided by Dr C.W. Young (Sloan-Kettering Memorial Cancer Center, New York, NY) who obtained them from Pharmatalia. The daunorubicin was purchased from Shands Hospital Pharmacy, Gainesville, FL, in the form of 20 mg vials of Cerubidine (Ives, New York, NY) which were reconstituted with 4 ml of deionized water. Stock solutions of the drugs were prepared in water with the exception of CN which was dissolved in methanol–water (50:50, v/v). HPLC grade organic solvents and ACS grade KH_2PO_4 were obtained from Fisher Scientific, Fair Lawn, NJ. All other solvents and chemicals were reagent grade, obtained from Fisher. Deionized water was used throughout. Silanized glassware was used for all solutions containing the anthracycline antibiotics.

Liquid chromatography

The liquid chromatograph was comprised of a Constametric IIG pump (LDC, Riveira Beach, FL), a manual injection valve (Negretti and Zamba (Aviation), Southampton, UK) fitted with a 200 μl loop, an SSI high pressure filter (Rainin Instruments, Woburn, MA) positioned between the injection valve and the column, a Rheodyne 3-way slider valve positioned between the column and the detector, a BioAnalytical Systems Model LC-4A amperometric detector (BAS, West Lafayette, IN) and a Fisher Recordall 5000 series strip chart recorder. Two analytical columns, ODS Hypersil (3 μm, 75 × 4.6 mm i.d., Shandon Southern, Sewickley, PA) and Zorbax ODS (5 μm, 75 × 4.6 mm i.d., DuPont, Wilmington, DE) were packed using the upward slurry technique [13]. The electrochemical detector was equipped with a TL-5A thin layer flow cell. The working electrode was glassy carbon and the reference electrode was Ag/AgCl.

The chromatographic conditions were optimized by investigating the effects of mobile phase composition on resolution and analysis time. The optimum mobile phase for the analysis of the six compounds of interest, with either column, was acetonitrile–isopropanol–0.1 M KH_2PO_4 (pH 4.5) (25:3:72, v/v). The aqueous portion of the mobile phase was filtered under vacuum through a 0.45 μm Millipore filter and degassed by sonication prior to use.

Cyclic voltammetry

Cyclic voltammetry was performed on the compounds of interest using a CV-IA cyclic voltammeter (BAS) and a MCA microcell (50 μl) assembly (BAS). The solute concentration was approximately 10^{-4} M in KH_2PO_4/H_3PO_4 buffer (0.1 M, pH 4.0) and a scan rate of 50 mV s^{-1} was used. The working electrode was carbon paste, the counter electrode was platinum wire and the reference electrode was Ag/AgCl. The recording

system was a digital voltammeter and an X–Y recorder, model RE0074 from Princeton Applied Research, Princeton, NJ.

Clinical study

4′-Deoxydoxorubicin (52 mg) was administered intravenously to a patient suffering from neoplastic disease. Blood samples (about 5 ml) were taken immediately after the end of the infusion and then after the following periods of time: 5, 10, 15, 30 and 45 min and 1, 2, 4, 6, 12, 24, 30 and 48 h. The blood samples were collected in heparinized tubes, centrifuged and the plasma was frozen (−20°C) prior to analysis.

Sample preparation

An appropriate volume of internal standard solution (Table 1) was added to 1 ml of plasma in a 15 ml teflon lined screw capped test tube. For the analysis of DOX, EPI and DMDR the pH of the plasma was adjusted to 8.4 by the addition of 100 μl of borate buffer (0.1 M, pH 9.0). For the analysis of DEOXY and CN the pH was adjusted to 8.6 by the addition of 120 μl of borate buffer. Chloroform (10 ml) was added to each tube which was then vortexed for 10 min. After centrifugation at 2000 rpm for 10 min, the aqueous layer was aspirated. The chloroform layer was transferred to a second 15 ml test tube and the solvent evaporated to dryness at 40°C under a gentle stream of nitrogen.

Table 1
Summary of chromatographic conditions for the analysis of anthracycline antibiotics by HPLC-EC

Analyte*	Internal standard	Stationary† phase	Flow rate (ml/min)	k'‡ Analyte	k'‡ Internal standard
DOX	EPI	Zorbax ODS (5 μm)	0.7	4.62	6.23
EPI	DOX	Zorbax ODS (5 μm)	0.7	6.23	4.62
DEOXY	DNR	Zorbax ODS (5 μm)	1.1	5.84	9.28
DMDR	DNR	Zorbax ODS (5 μm)	1.6	15.02	9.28
CN	DNR	ODS Hypersil (3 μm)	0.8	14.66§ 16.67§	9.85

*See Fig. 1 for key.
†Column dimensions: 4.6 × 75 mm i.d. Mobile phase: acetonitrile–sopropanol–0.1 M KH_2PO_4 (pH 4.5).
‡$k' = t_r/(t_r - t_o)$, where t_r and t_o are the retention times of the solute and unretained compound, respectively.
§Separated diastereomers.

Residues containing DOX, EPI or DEOXY were redissolved in 210 μl of 0.2 M H_3PO_4 (pH 2.0), vortex mixed for 15 s and shaken for 30 s with 1 ml of chloroform. Residues containing DMDR or CN were redissolved in 210 μl of 0.1 M KH_2PO_4 (pH 4.5), vortex mixed for 15 s and shaken for 30 s with 1 ml of *n*-hexane. After centrifugation at 2000 rpm for 2 min, 200 μl of the aqueous phase was injected onto the HPLC column.

Calibration curves

For each drug studied, two calibration curves were constructed, one spanning a high concentration range of drug (0–4.0 μg ml^{-1}) and one spanning a low concentration range of drug (0–100 ng ml^{-1}). For the high concentration calibration curves, 1 ml of blank plasma was spiked with 0.2, 0.4, 0.8, 1.6, 2.4, 3.2 and 4 μg of the analyte plus 1 μg of internal standard (Table 1). For the low concentration range calibration curve, 1 ml of plasma was spiked with 10, 20, 40, 60, 80 and 100 ng of analyte plus 20 ng of internal standard (Table 1). Each spiked plasma sample was extracted in an identical manner to that described for the samples. In all cases, the relationships between peak height ratio and the concentration of drug injected were linear ($r > 0.998$) with zero intercepts.

Results and Discussion

Assay development

Despite the wide range in hydrophobicities of the compounds studied it was possible to develop a single set of chromatographic conditions which was suitable for their analysis (Fig. 2). The chromatography of the anthracycline antibiotics was optimized in terms of resolution from interfering endogenous substances, peak shape and overall analysis times by investigating the effects of mobile phase composition and flow rate. Initially, binary mobile phases of methanol–phosphate buffer (0.1 M, pH 4.5) or acetonitrile–

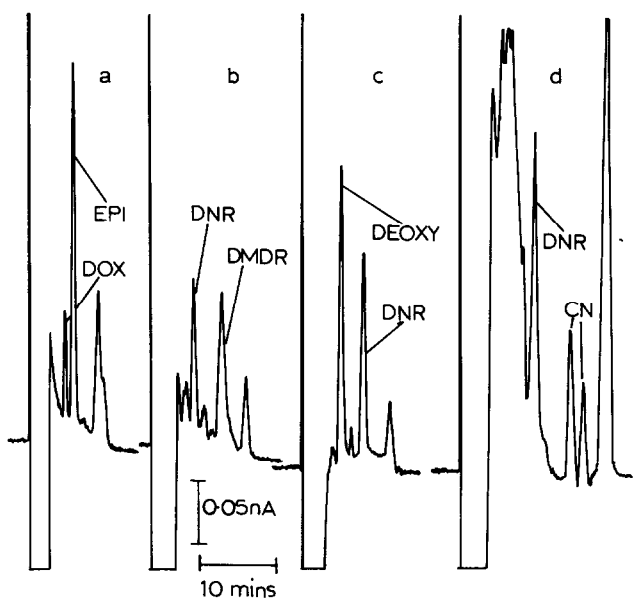


Figure 2
Chromatograms from the analysis of DOX (a), DMDR (b), DEOXY (c) and CN (d) in plasma. EPI is the internal standard in (a) and DNR is the internal standard in (b), (c) and (d). It should be noted that DOX may be used as internal standard for EPI. Although not validated in this study, either DMDR or DEOXY would be suitable internal standards for DNR. See Table 1 for chromatographic details. The solute concentrations were: 20 ng ml^{-1} EPI; 5 ng ml^{-1} DOX; 20 ng ml^{-1} DNR; 25 ng ml^{-1} DMDR; 12 ng ml^{-1} DEOXY; and 25 ng ml^{-1} total CN.

phosphate buffer were investigated and both combinations were found to be suitable for the chromatography of the compounds of interest. However, acetonitrile was preferred to methanol since it could be used at lower concentrations which resulted in lower background currents and less noise from the electrochemical detector. In addition, it was also found that the inclusion of a small concentration of isopropanol (3%) further reduced the noise and also produced sharper peaks. Thus the optimum mobile phase was found to be acetonitrile–isopropanol–0.1 M phosphate buffer pH 4.5 (25:3:72, v/v). The two columns, ODS Hypersil and Zorbax ODS, investigated were found to give virtually identical retention characteristics. However, the 3 μm ODS Hypersil column gave slightly sharper and more symmetrical peaks than the 5 μm Zorbax ODS column. In general, the use of short columns (7.5 cm) permitted the rapid analysis of the compounds of interest, with the exception of CN, at relatively low back pressures (500–1000 psi). Changing from the more conventional 15 cm columns to columns of 7.5 cm in length permitted the analysis times to be halved and the sensitivity to be doubled. Further decreases in column length to 5 cm resulted in poor peak shape and reductions in sensitivity. These effects may be attributed to increases in the relative contribution of extra-column effects to the overall band broadening. In addition, by their very nature, amperometric detectors have relatively slow response characteristics and cannot accommodate very rapidly eluting peaks. In the case of CN, an analysis time of 20 min was required for the separation of its two diastereomers. The analysis time for CN could be reduced by increasing the organic modifier content of the mobile phase or increasing the flow rate. However, this could only be achieved at the expense of incomplete resolution of its diastereomers.

The chromatographic behaviour of the anthracycline antibiotics was found to be particularly sensitive to the pH of the aqueous component in the mobile phase, with a pH of 4.5 being optimal. Higher or lower pH values tended to produce pronounced peak tailing. In addition, the nature of the buffer added to the mobile phase had an effect on the chromatographic behaviour. For example replacement of 0.1 M KH_2PO_4 with a 0.1 M acetate buffer of the same pH gave similar retention characteristics but very broad peaks.

The retention of the anthracycline antibiotics was found to be related to solute hydrophobicity and it was possible to calculate some of the contributions of the functional groups, τ, (log (k'_2/k_1), ref. 14) to retention. The value of τ for the alcoholic OH at position 14 (-0.30) is obtained from the retention data for DOX and DNR and the contribution ($\tau = -0.10$) of the same functional group at the 4′ position on the amino sugar is obtained from the data for DOX and DEOXY (Table 1). Interestingly, the two epimers of doxorubicin were well separated ($\tau = 0.13$) such that one could be used as an internal standard for the other. The contribution of the methoxy group ($\tau = 0.21$) in the 4 position is obtained from the retention data for DMDR and DNR. Replacement of the 3′-amino group by the hydrophobic 3-cyanomorpholino group caused a tremendous increase in retention. The two isomers of 3′-CN were separated indicating a difference in the hydrophobic contributions of the cyanomorpholine groups to retention ($\tau_1 = 0.50$ and $\tau_2 = 0.56$).

The optimum conditions for amperometric detection of the compounds of interest were determined from hydrovoltammograms (Fig. 3) in which the detector response (peak height) was determined as a function of the applied oxidation potential. The profiles of the hydrovoltammograms for each of the compounds were very similar (Fig. 3), indicating that it is the same functional group which is oxidized and gives rise to the

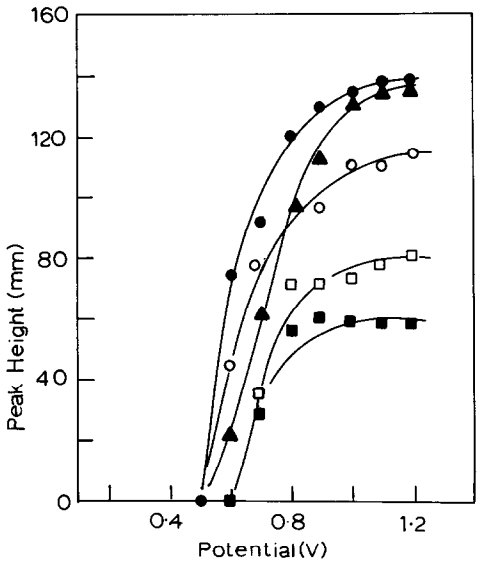


Figure 3
Hydrovoltammograms for five anthracycline antibiotics. See Table 1 for chromatographic conditions. Key: DOX (●); DEOXY (○); EPI (▲); DMDR (□); and CN (■).

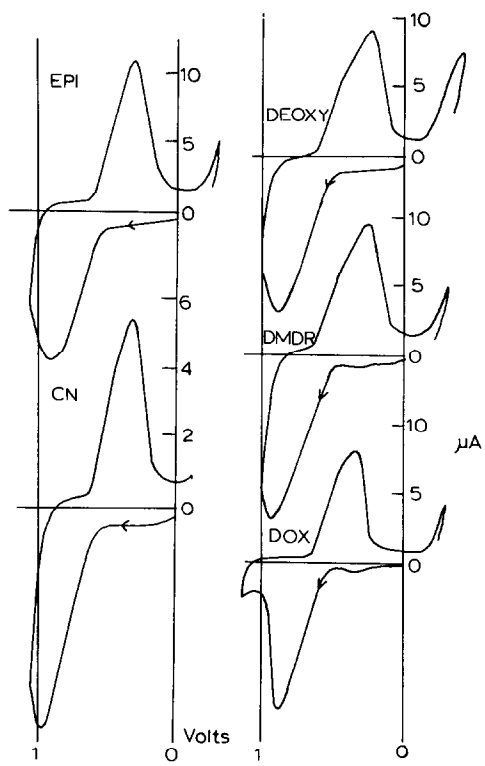


Figure 4
Cyclic voltammograms of five anthracycline antibiotics. Scan rate: 50 mV s^{-1}. Solute concentration: *ca* 10^{-4} M.

measured diffusion current. This conclusion was supported by the cyclic voltammograms (Fig. 4) for each of the compounds which were very similar, showing anodic peaks between 0.7 and 0.9 V and cathodic peaks between 0.4 and 0.6 V at pH 4.0 in the oxidation mode (Fig. 4). For chromatographic analysis, an applied potential of 0.8 V gave the best signal to noise ratio and was used for the detection of all the compounds studied. It is reasonable to assume that oxidation of one of the aromatic hydroxyl groups on the anthracycline moiety occurs in the detector. The half-wave potential for the oxidation of the anthracyclines is higher than that seen for phenolic compounds such as the catecholamines [15]. Consequently detection of the anthracyclines is less selective than for the catecholamines and greater attention to detail is necessary in the sample preparation and chromatographic optimization for the HPLC-EC of these compounds. However, using the procedures described here, detection limits of 200 pg are possible. This permitted the anthracyclines to be quantified in plasma at concentrations down to 1 ng ml^{-1} which is equal to or lower than the limits of quantification by HPLC using fluorescence detection [6–10]. The coating of the glassy carbon electrode by irreversibly bound plasma constituents was minimized by the use of a 3-way slider valve positioned between the column and the flow cell. The materials eluting before the peaks of interest were vented to waste. This significantly reduced the frequency with which the surface of the working electrode had to be regenerated by polishing. Also, it prevented excessive contamination of the mobile phase which generally was recycled. Recycling of the mobile phase resulted in lowered background current and detector noise.

Selective isolation of the anthracyclines from plasma was achieved by an alkaline extraction into chloroform, followed by back extraction into an acidic aqueous solution. The remaining interferences were removed by shaking the final aqueous aliquot with an organic solvent (*n*-hexane or chloroform). Since the anthracyclines are amphoteric their extraction into chloroform was highly pH dependent. The highest recovery of DOX, EPI and DMDR into chloroform was achieved at a pH of 8.4. A pH of 8.6 was preferred for the extraction of DEOXY and CN. Removal of interferences from acidified extracts containing DOX, EPI or DEOXY could be achieved at pH 2.0 by shaking with chloroform. However, these conditions were unsuitable for the clean-up of the more hydrophobic DMDR and CN which partitioned into the chloroform, resulting in low recoveries. Clean-up of CN and DMDR was achieved by shaking with *n*-hexane at pH 4.5. In addition, a pH of 4.5 was needed for the clean-up of CN because both diastereomers were found to be unstable at pH 2.0.

Assay validation

The absolute recoveries of the extraction procedures were determined by comparing the peak heights of the drug extracted from spiked plasma (10 ng ml^{-1}) with the peak heights of the drug in aqueous solutions at the same concentration. The absolute recoveries ranged between 56.8% for DMDR and 83.2% for one of the two isomers of CN (Table 2).

The accuracy and precision of the procedures were determined at two concentrations (10 ng ml^{-1} and 1 μg ml^{-1}). Five 1 ml aliquots of blank plasma were spiked with either 10 ng or 1 μg of antibiotic and analysed using the described procedures. The relative standard deviation, used as a measure of precision, ranged between 1.22% for one of the isomers of CN at 10 ng ml^{-1} and 6.43% for DOX at 1 μg ml^{-1} (Table 3). The accuracy of the procedures, measured by the percentage of the actual concentration found, ranged between 94.5 and 106% (Table 3).

Table 2
Absolute recoveries of the anthracycline antibiotics*

Analyte†	Recovery (%)‡	RSD (%)§
DOX	62.5	5.2
EPI	59.8	4.4
DEOXY	76.3	7.4
DMDR	56.8	4.5
CN_1	83.2	5.1
CN_2	76.4	5.9

*See Table 1 for chromatographic conditions.
†See Fig. 1 for key. Concentration 10 ng ml^{-1} for each drug.
‡Peak height of the drug extracted from plasma compared with that of the drug in aqueous solution, expressed as a percentage.
§Relative standard deviation, $n = 5$.

Table 3
Accuracy and precision data for the analysis of anthracycline antibiotics by HPLC-EC*

Analyte†	Concentration (ng ml^{-1}) added	found	Accuracy‡ (%)	Precision§ (%)
DOX	1000	984	98.4	6.43
	10.0	10.50	105.0	1.23
EPI	1000	1044	104.4	5.23
	10.0	9.57	95.7	1.46
DEOXY	1000	995	99.5	6.46
	10.0	9.45	94.5	3.89
DMDR	800	795	99.4	1.65
	10.0	10.60	106.0	4.52
CN_1	420‖	421	100.2	2.38
CN_2	380‖	360	94.7	5.58
CN_1	5.30¶	5.40	101.9	1.22
CN_2	4.70¶	4.92	104.7	4.07

*See Table 1 for chromatographic conditions.
†See Fig. 1 for key.
‡Concentration found divided by concentration added, expressed as a percentage.
§Relative standard deviation, $n = 5$.
‖Total CN concentration was 800 ng ml^{-1}. Concentrations of the individual isomers were determined from their peak areas using an HP 3392A integrator.
¶Total CN concentration was 10 ng ml^{-1}. Concentrations of the individual isomers were determined from their peaks areas using an HP 3392A integrator.

Assay applicability

The applicability of the assay procedures to pharmacokinetic studies was demonstrated by the analysis of plasma samples taken from a cancer patient who had received 52 mg of DEOXY by intravenous infusion (Fig. 5). These samples had been assayed previously in another laboratory (Memorial Sloan-Kettering Cancer Center) using an

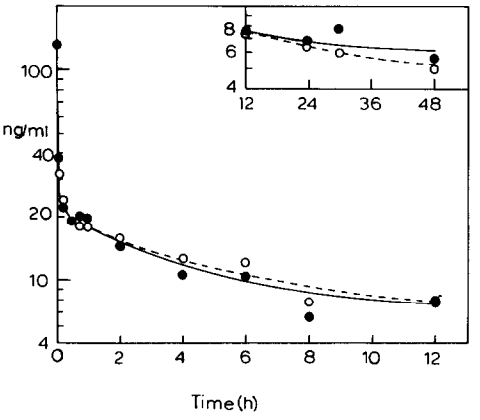


Figure 5
Plasma concentration–time profile of DEOXY following intravenous injection of 52 mg, determined by HPLC-EC (O---O) and HPLC-FL (●——●). The lines have been drawn according to equations (2) and (3). See Table 1 and ref. 6 for HPLC-EC and HPLC-FL conditions, respectively.

HPLC procedure [6] which employed fluorescence detection (HPLC-FL). It can be seen from Fig. 5 that the agreement between the data obtained by HPLC-EC and HPLC-FL is excellent. The relationship, obtained by linear regression, between the concentrations found by HPLC -EC (C_e) and those found by HPLC-FL (C_f) is given in equation 1:

$$C_e = 0.990\ C_f - 1.04,\ r^2 = 0.990. \quad (1)$$

The data obtained by HPLC-EC and HPLC-FL was fitted to a three compartment open body model by non-linear least squares regression (SASNLIN, version 8.24, SAS Institute, Cary, NC). The data by HPLC-FL and HPLC-EC satisfy equations (2) and (3), respectively.

$$C_f = 98.9e^{-0.35t} + 15.5e^{-6.45\times10^{-3}t} + 7.81e^{-8.11\times10^{-5}t} \quad r^2 = 0.998 \quad (2)$$

$$C_e = 101.9e^{-0.44t} + 13.5e^{-3.81\times10^{-3}t} + 7.29e^{-1.10\times10^{-4}t} \quad r^2 = 0.999. \quad (3)$$

Conclusions

Anthracycline antibiotics of widely differing hydrophobicities may be analysed in plasma by HPLC-EC using the same chromatographic conditions. The only differences in the assay procedures for the drugs studied are slight modifications in the extraction procedures and flow rates of the eluent. Since all the compounds may be eluted with the same mobile phase, any one drug may be used as an internal standard for the analysis of a second. The procedures are accurate and precise, and the sensitivity is sufficient to allow the pharmacokinetic investigation of deoxydoxorubicin following intravenous administration of this potent anthracycline antibiotic. The agreement between HPLC using electrochemical and fluorescence detectors is excellent. The limit of detection for the compounds studied was between 1 and 2 ng ml^{-1} which compares favourably with other methods using fluorescence detection [6–10] and is better than that reported previously [11, 12] for electrochemical detection of these types of compounds. With the application of more sophisticated dual electrode detectors [16] and the use of difference

mode detection described recently by Lunte *et al.* [17] the limits of detection of anthracycline antibiotics by HPLC-EC may be reduced so that they significantly surpass those achievable by other techniques.

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