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Note

Assay of doxorubicin and 4'-epidoxorubicin by reversed-phase ion-pair chromatography

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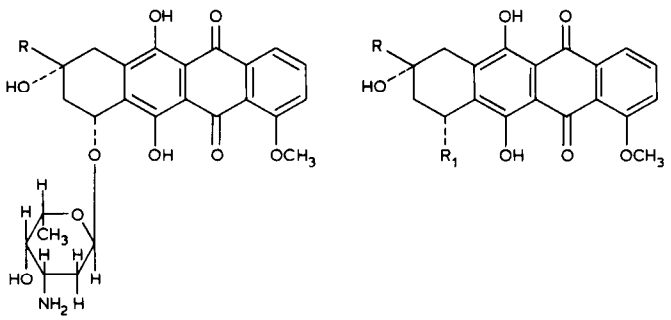
Anthracyclines form a group of structurally related antibiotics used as anti-neoplastic agents. The basic anthracycline structure consists of a tetracyclic quinoid moiety glycosidally linked to the amino sugar daunosamine. Daunorubicin (daunomycin) and doxorubicin (adriamycin) are used in the treatment of acute leukaemia, certain lymphomas and solid tumours. Recently, a less toxic derivative of doxorubicin, 4'-epidoxorubicin, has been introduced. Pharmaceutical preparations of doxorubicin may contain traces of several related substances which include daunorubicin and the aglycones daunorubicinone and doxorubicinone, as well as other minor impurities (Fig. 1). It is necessary to determine both the content and purity of doxorubicin preparations as treatment depends on an accurate dosage schedule, in order to minimise the risk of cardiac toxicity associated with the drug. The aglycone content should be restricted, as these impurities are mutagenic, but lack antitumour activity[1].

Both normal-phase[2] and reversed-phase[3] high-performance liquid chromatography have been used for the determination of anthracycline content. Reversed-



R		R_1	
$COCH_3$	Daunorubicin	OH	Daunorubicinone
$CH(OH)CH_3$	Daunorubicinol		
$COCH_2OH$	Doxorubicin	OH	Doxorubicinone
$CH(OH)CH_2OH$	Doxorubicinol		

Fig. 1. Structural formulae of doxorubicin and related anthracyclines.

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phase systems have been proposed for the separation and quantitation of daunorubicin, doxorubicin and their aglycones in the pharmaceutical preparations[4] and for the separation of several anthracycline derivatives and their metabolites in biological samples[5–8]. Recently, a new isocratically reversed-phase ion-pair chromatographic system, which resolved eight anthracycline derivatives, has been described[9]. The work described here details the application of this system to the assay of doxorubicin and 4′-epidoxorubicin.

EXPERIMENTAL

Materials

Acetonitrile (HPLC-S grade, Rathburn Chemicals, Walkerburn, U.K.), sodium dodecyl sulphate (Fisons Scientific, Loughborough, U.K.) and analytical grade solvents and reagents (BDH, Poole, U.K.) were used. The anthracycline antibiotics: doxorubicin (Adriamycin) and 4′-epidoxorubicin (Pharmorubicin) and their derivatives 13-dihydrodoxorubicin (doxorubicinol), doxorubicinone, 11-desoxydoxorubicin, daunorubicin (daunomycin), 13-dihydrodaunorubicin (daunorubicinol, duborimycin) and daunoribicinone were very kindly supplied by Mark Griso of Farmitalia Carlo Erba, Barnet, U.K.

High-performance liquid chromatography

The chromatographic system consisted of a dual reciprocating pump (Consta Metric III, Laboratory Data Control, FL, U.S.A.) and Rheodyne injector equipped with a 20-μl loop (Model 7125, Rheodyne Inc., Berkeley, CA, U.S.A.). A pre-column (40 × 4.6 mm) packed with general purpose grade Spherisorb ODS was placed between the pump and the injector; a column (250 × 4.6 mm) packed with Hypersil ODS, 5 μm, was used for the separation of the anthracyclines. The mobile phase consisted of water containing 0.033 *M* phosphoric acid and 0.039 *M* sodium dodecyl sulphate adjusted to pH 2.4 with 4 *M* sodium hydroxide–acetonitrile (100:76). The mobile phase was filtered through a glass microfibre filter and degassed prior to use. The flow-rate was 1 ml/min. The anthracyclines (100 μg/ml) were dissolved in 40% methanol. The eluent was monitored by absorbance at 254 nm (0.5 a.u.f.s.) or 480 nm (0.25 a.u.f.s.) with a variable-wavelength spectrophotometer (Model CE 272, Cecil Instruments, Cambridge, U.K.). Fluorescence detection was also tried with excitation 467 nm, emission 570 nm (Model 1000 M Perkin Elmer, Beaconsfield, U.K.). The peak areas were measured with a computing integrator (Model 301, Laboratory Data Control).

RESULTS AND DISCUSSION

Good resolution of the potential anthracycline impurities in doxorubicin and 4′-epidoxorubicin was achieved when an eight-component mixture was chromatographed (Fig. 2). The retention times, capacity factors and resolution values observed under these conditions are summarised in Table I.

Since the peaks were sharp and well separated, the quantitative determination of doxorubicin and 4′-epidoxorubicin could be based on peak height measurements. A linear relationship between peak height and anthracycline concentration was ob-

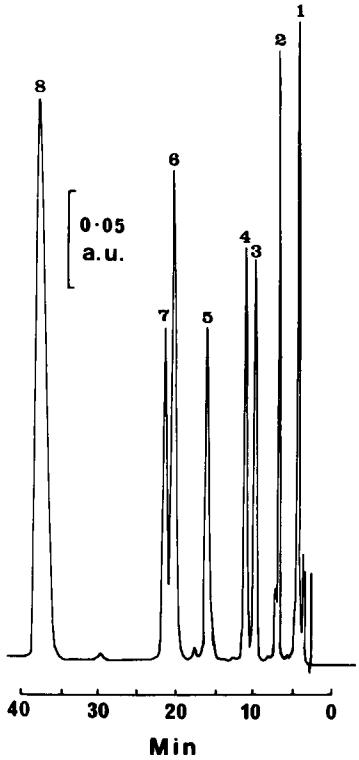

Fig. 2. Chromatogram of a mixture of anthracyclines. Column: Hypersil ODS, 250 × 4.6 mm (5 μm). Eluent: 0.033 *M* phosphoric acid and 0.039*M* sodium dodecyl sulphate, pH 2.4–acetonitrile (100:76), flow-rate 1 ml min^{-1}, detection 254 nm. Identification: 1 = doxorubicinone; 2 = daunorubicinone; 3 = doxorubicinol; 4 = desoxydoxorubicin; 5 = doxorubicin; 6 = 4′-epidoxorubicin; 7 = daunorubicinol; 8 = daunorubicin.

TABLE I

RETENTION TIME (t_R), CAPACITY RATIO (k') AND RESOLUTION VALUE (R_S) OF ADJACENT PEAKS FOR THE ANTHRACYCLINES

$k' = (t_R - t_o)/t_o$ where t_R = retention time of the compound and t_o = retention time of solvent front; $R_S = 2(t_{R_2} - t_{R_1})/(w_2 + w_1)$, where t_{R_1}, t_{R_2} = retention time of compounds 1 and 2; and w_1, w_2 = peak width at baseline of compounds 1 and 2. Chromatographic conditions as in text.

Anthracycline	t_R *(min)*	k'	R_S
Doxorubicinone	4.00	0.65	–
Daunorubicinone	6.48	1.68	4.95
Doxorubicinol	9.75	3.05	4.36
Desoxydoxorubicin	10.88	3.54	1.13
Doxorubicin	15.95	5.61	4.50
4′-Epidoxorubicin	20.38	7.44	3.54
Daunorubicinol	21.43	7.89	0.84
Daunorubicin	37.66	14.59	8.11

tained in the range used, even though an internal standard was not employed. Linear regression analysis of the dose-responses from 20–200 μg/ml measured at 254 nm and 480 nm yielded correlation coefficients of 0.9998 summarised in Table II.

TABLE II

DOSE RESPONSE CALIBRATION OF DOXORUBICIN AND 4'-EPIDOXORUBICIN ANALYSED BY METHOD OF LEAST SQUARES

Chromatographic conditions as in text, peak heights measured for concentrations between 20–200 μg ml^{-1} at 254 nm and 480 nm.

Anthracycline	*Wavelength (nm)*	*Regression equation*	*Correlation coefficient*
Doxorubicin	254	$y = 85.8145x + 1.8526$	0.9998
	480	$y = 0.3258x + 1.2210$	0.9998
4'-Epidoxorubicin	254	$y = 56.1829x + 0.9305$	0.9997
	480	$y = 65.6230x + 0.7868$	0.9998

The most sensitive means of detecting all the anthracyclines examined was absorbance at 254 nm. At that wavelength the absorbance of doxorubicin is approximately twice that at 480 nm $E^{1\%}_{1cm}$ at 254 nm = 440, $E^{1\%}_{1cm}$ at 480 nm = 225). For desoxydoxorubicin fluorescence and absorbance at 480 nm was very low ($E^{1\%}_{1cm}$ at 254 nm = 327, $E^{1\%}_{1cm}$ at 480 = 26). For this reason the wavelength 254 nm was chosen to detect and measure the anthracyclines under investigation.

The reproducibility of the system was determined on six replicate injections, giving a relative standard deviation of 0.92%, 1.39% and 0.95% respectively for doxorubicin, 4'-epidoxorubicin and a mixture of 4'-epidoxorubicin and lactose. Six independent assays of doxorubicin done on six separate occasions gave a relative standard deviation of 1.80%.

For quantitative measurements the standard solution was chromatographed before and after duplicate injections of the test solution. Samples of doxorubicin and 4'-epidoxorubicin were assayed against the corresponding working standard on three separate occasions and compared with the claimed content (Table III). A sample of doxorubicin which had been stored at 4°C for eight years, assayed at 89.20%.

TABLE III

ASSAY DATA FOR DOXORUBICIN AND 4'-EPIDOXORUBICIN

Assay values are presented as the mean of three independent assays.

Sample	*Claimed content (%)*	*Amount found (%)*	*± Limits (P = 0.95)*	*Recovery (%)*
Doxorubicin 1	90.00	90.01	2.59	100.01
2	97.75	97.52	0.40	99.76
3	98.20	99.60	1.59	101.42
4	97.60	98.06	1.56	100.41
4'-Epidoxorubicin 1	82.80	81.55	2.15	98.49
2	100.00	100.24	1.55	100.24

The injectable prepartions of both doxorubicin and 4'-epidoxorubicin contain 5 parts by weight of lactose, therefore assay of the antibiotics in the presence of lactose was investigated. Aqueous methanol, 40% (v/v), was found to be a suitable diluent for the anthracyclines and the lactose. Varying the amount of lactose had no effect on either the recovery of the antibiotic or the accuracy of the assay (Table IV). Injectable preparations were assayed against their corresponding standards in the absence of lactose (Table V). Samples B and C had reached their expiry date in 1980, whereas preparations A and D were of recent manufacture.

TABLE IV

EFFECT OF LACTOSE ON RECOVERY AND THE ASSAY OF DOXORUBICIN AND 4'-EPI-DOXORUBICIN

Recovery of solutions of doxorubicin and 4'-epidoxorubicin 75 $\mu g\ ml^{-1}$ containing lactose; 200; 400 and 700 $\mu g\ ml^{-1}$ compared with the corresponding solution without lactose. Assay of solutions of doxorubicin and 4'-epidoxorubicin 75.0; 73.5 and 76.5 $\mu g\ ml^{-1}$ (98 and 102% of claimed content) containing lactose 375 $\mu g\ ml^{-1}$). Chromatographic conditions as in text, detection at 254 nm.

	Concentration ($\mu g\ ml^{-1}$)	*Recovery (%)*	
		Doxorubicin	*4'-Epidoxorubicin*
Lactose	200	100.00	97.70
	400	99.87	100.77
	700	99.86	100.89
Antibiotic	75.0	98.62	100.17
	73.5	97.37	99.80
	76.5	100.92	101.60'

TABLE V

ASSAY DATA FOR DOXORUBICIN AND 4'-EPIDOXORUBICIN

Assay values are presented as the mean of three independent assays.

Sample	*Labelled content (mg)*	*Amount found (mg)*	*± Limits (P = 0.95)*	*Recovery (%)*
Doxorubicin A	50	49.35	1.48	98.70
B	50	48.73	1.60	97.46
C	10	9.70	0.34	97.04
4'-Epidoxorubicin D	50	49.72	1.65	99.44

Determination of the relative composition of doxorubicin and 4'-epidoxorubicin bulk materials revealed the presence of less than 1.0% related substances in the sample of 4'-epidoxorubicin and between 0.8 and 2.0% in the recent samples of doxorubicin. However, the eight year old sample of doxorubicin contained 4.9% related substances, which included 1.13% doxorubicinone and 1.58% daunorubicin.

The reversed-phase ion-pair chromatographic system proved to be suitable for the quantitative determination of doxorubicin and 4'-epidoxorubicin in dosage and bulk preparations. The assay was sufficiently precise not to require an internal standard. The method was also suitable for the qualitative assessment of both antibiotics.

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