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DETERMINATION OF ANTHRACYCLINE PURITY IN PATIENT SAMPLES AND IDENTIFICATION OF IN VITRO CHEMICAL REDUCTION PRODUCTS BY APPLICATION OF A MULTI-DIODE ARRAY HIGH-SPEED SPECTROPHOTOMETRIC DETECTOR

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SUMMARY

We describe the application of a high-speed spectrophotometric detector and high-performance liquid chromatography to the determination of anthracycline purity in extracted patient specimens and to the identification of chemical reduction products. Blood contained pure anthracyclines whilst in urine, tissue and tumour there was evidence of co-eluting endogenous peaks and complexation. Aerobic reduction yielded two main products: a C_{13} alcohol and a fully reduced, non-fluorescent, yellow hydroquinone. Anaerobic reduction in the presence of DNA yielded a 7-deoxyaglycone metabolite end product instead of the fully reduced hydroquinone. Eight other separate chromatographic species were identified, all of which showed unique absorbance characteristics, having a visible λ_{max} at 530 nm and being coloured purple/blue.

INTRODUCTION

The anthracyclines represent an important class of anti-cancer drugs because of their broad spectrum of clinical activity [1]. Daunorubicin (DNR) and adriamycin (ADR) are the prototypes. However, problems with dose-dependent cardiotoxicity and resistance as well as the high systemic toxicity normally

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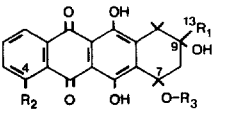
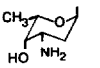
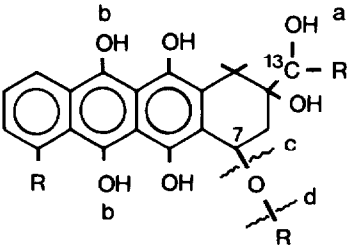


	R_1	R_2	R_3
Adriamycin (ADR)	$-\overset{O}{\overset{\|}{C}}-CH_2OH$	$-OCH_3$	
Daunorubicin (DNR)	$-\overset{O}{\overset{\|}{C}}-CH_3$	$-OCH_3$	
4'-Deoxydoxorubicin (4'-DOX)	$-\overset{O}{\overset{\|}{C}}-CH_2OH$	$-OCH_3$	
4-Demethoxydaunorubicin (4-DMDNR)	$-\overset{O}{\overset{\|}{C}}-CH_3$	H	

B



a) C_{13} carbonyl reduction : alcohol

b) Quinone reduction : hydroquinone

c) Reductive cleavage of C7 glycoside bond 7- deoxyaglycone

d) Hydrolytic cleavage of C7 glycoside bond . 7- hydroxyaglycone

Fig. 1. (A) Structures of the four anthracyclines studied. (B) Structures of the anthracycline metabolites studied.

associated with cytotoxic drugs have stimulated the search for new derivatives with improved therapeutic index. 4-Demethoxydaunorubicin (4-DMDNR) is a potent orally active analogue of DNR which has recently completed phase I and II clinical trials [2]. 4'-Deoxydoxorubicin (4'-DOX) is a promising new anthracycline entering clinical trials which has a broad spectrum of anti-tumour activity in animals but appears to be non-cardiotoxic in man [3] (Fig. 1).

Whilst the in vivo mode of action of any one anthracycline has not been fully established, it is unlikely that all act by the same mechanism(s) or at the same target site(s) in the cell. Several different modes of action have been proposed for ADR, each of which may operate under different circumstances and conditions. Native ADR binds avidly to DNA by intercalation resulting

in an inhibition of its template functions [4]. It can undergo aerobic bioreduction to a semi-quinone free radical which induces DNA strand damage [5, 6] and anaerobic bioreduction to a series of reactive aglycone intermediates which bind covalently to DNA [7, 8]. It has also been demonstrated to be cytotoxic without entering cancer cells [9]. During anaerobic bioreduction fully reduced ADR hydroquinone is an intermediate. A 7-deoxyaglycone metabolite is an end product of both forms of reduction.

At all levels of anthracycline research high-performance liquid chromatography (HPLC) has been applied extensively and coupled to a variety of detection methods. These include radioimmunoassay, fixed-wavelength UV absorbance, fluorescence and electrochemical detection [10—13]. Of all these techniques fluorescence detection has been most widely employed because of high sensitivity and selectivity. Conventionally, each of these detection methods offers little or no qualitative information and as a consequence is subject to errors in quantitation. Radioimmunoassay is unable to discriminate between parent drug and metabolites. Fluorescence quenching due to complexation with biomolecules and co-elution with impurities could lead to a serious underestimation of concentration. For the same reasons, UV absorbance and electrochemical detection could lead to large errors. All techniques may not be able to identify important biotransformed intermediates.

We have previously described the application of a multi-diode array detector to HPLC analysis of ADR incorporated into albumin microspheres [14]. In this report we describe its broader application to the analysis of anthracyclines in blood, urine, tissue and tumour specimens from patients and to the identification of chemically reduced intermediates.

EXPERIMENTAL

Reference compounds and reagents

Pure adriamycin · HCl, 4-demethoxydaunorubicin and 4′-deoxydoxorubicin were all kindly supplied by Farmitalia (Milan, Italy). Daunorubicin, for administration to patients, was from May and Baker (Dagenham, U.K.). Pure adriamycinol · HCl, adriamycin 7-deoxyaglycone, 4-demethoxydaunorubicinol · HCl and 4-demethoxydaunorubicin 7-deoxyaglycone were also from Farmitalia. 7-Hydroxyaglycones were synthesised from their parent drugs and their C_{13} alcohols by acid catalysed hydrolysis with 1 *M* hydrochloric acid at 40°C for 1 h. 7-Deoxyaglycones were synthesised by catalytic hydrogenation using a poisoned palladium $Pb/BaCO_3$ catalyst (B.D.H., Poole, U.K.).

Calf thymus DNA (type I, sodium salt) was from Sigma (Poole, U.K.) and sodium borohydride was from B.D.H. (AnalaR grade). All other reagents and chemicals were of the highest grade available commercially.

Chromatographic conditions

HPLC equipment, the method of separation of ADR and its metabolites and the method of separation of 4′-DOX and its metabolites have already been described in detail [15, 16]. DNR was determined using the same conditions

as 4′-DOX. 4-DMDNR was determined essentially according to the method described for 4′-DOX except that the mobile phase consisted of methanol—5 m*M* phosphoric acid (80:20), pH 3.2 eluting at a flow-rate of 2 ml/min. The multi-diode array detector was configured as before [14].

Extraction of anthracyclines

All anthracyclines and their metabolites were extracted from serum, plasma, urine and aqueous solutions with chloroform—propan-2-ol (2:1) by the rapid method previously described [15]. ADR and metabolites were extracted from homogenised tissue pretreated with silver nitrate (33%, w/v, 0.2 ml per ml homogenate) by an adapted method [17].

Patient samples

ADR blood samples and urine collections were from patients receiving 40 mg/m^2 as part of chemotherapy for advanced malignant disease. 4′-DOX blood samples and urine collections were from patients receiving 30 mg/m^2 as a single agent in a phase II clinical trial. 4-DMDNR blood samples and urine collections were from patients receiving 50 mg/m^2 orally (as three approximately equal doses every 8 h) in a phase II clinical trial for advanced small cell lung cancer. Liver, tissue and tumour specimens were obtained from open biopsies and resections from patients undergoing surgery for gastric and colo/rectal cancer. A low dose of ADR (25 mg/m^2) was administered intravenously approximately 30 min before resection.

Chemical reduction of 4′-deoxydoxorubicin, adriamycin and 4-demethoxydaunorubicin under aerobic conditions

Free anthracycline base (10 mg) was reconstituted in 20 ml methanol and 1 ml of 3.8% sodium borohydride (38 mg/ml). The reduction was allowed to proceed for 5 min and was then stopped by addition of two drops of glacial acetic acid [18]. The reaction mixture was aerated by bubbling through air for 24 h at 4°C in the dark. The end product was analysed directly.

Anaerobic reduction of adriamycin in the presence of DNA

ADR (1 mg) was added to 10 ml of 5 m*M* phosphate buffer, pH 7.0, in a 25-ml rubber-lined screw-capped test tube. Nitrogen gas was bubbled through the mixture and the cap was screwed on tightly to retain a nitrogen atmosphere. Tubes were covered in aluminium foil to protect from light. At three consecutive 5-min intervals, 400 μg of borohydride were added to the ADR solution [19]. After the first addition of borohydride 1 mg of DNA was added. Each time the cap was removed nitrogen gas was bubbled through the mixture before it was replaced. After the last addition of borohydride the mixture was incubated overnight at room temperature and in the dark before direct analysis of the end product.

RESULTS AND DISCUSSION

A diode array detector can monitor chromatographic peaks at several different wavelengths simultaneously, enabling transformed products with

different absorbance characteristics from the parent drugs to be identified. Comparison of peak symmetry at two different wavelengths can confirm chemical purity. However, because the diode array detector measures absorbances at all wavelengths from 190 to 600 nm (in 2-nm steps) every 10 ms, it can effectively scan a chromatographic peaks UV—visible spectrum several times as it elutes from an HPLC system. If an anthracycline peak contains a complex, it can be identified by a well defined spectral shift. If it contains an impurity it can be identified by detection of its UV—visible spectrum and attenuation of the anthracycline's own spectrum. Acquisition of multi-spectral data is a unique function of the diode array detector, and it is this ability which enables qualitative HPLC analysis to be performed.

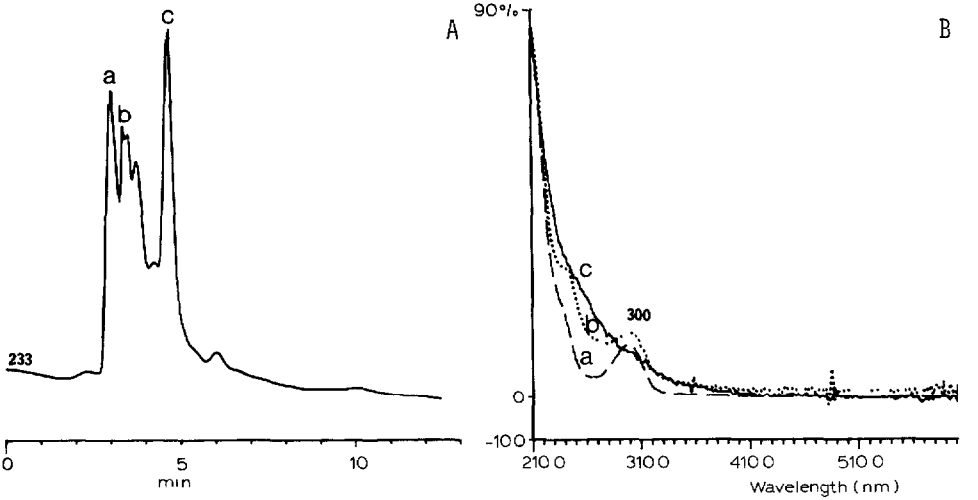


Fig. 2. (A) Chromatogram of an aqueous solution of adriamycin maintained at room temperature for several weeks. Peaks were monitored at 233 nm. Full scale attenuation was 50 mA.U. Three main peaks were resolved (peaks a, b and c). Chromatographic conditions: mobile phase was 5 m*M* (final concentration) phosphoric acid in propan-2-ol, pH 3.2 (74:26) and the stationary phase was μBondapak C_{18} (250 mm × 4.6 mm I.D.). Elution was isocratic at a flow-rate of 1.2 ml/min. (B) UV—visible spectra of peaks a, b and c from A.

Fig. 2A and B illustrates the usefulness of the diode array detector. An aqueous solution of ADR maintained at room temperature for several weeks was chromatographed and the peaks resolved scanned. Considerable degradation of ADR would be expected to occur during this period of time [15]. Fig. 2A shows that several ADR-related peaks were detected at 233 nm, the λ_{max} of ADR. Spectral analysis demonstrated that none of these peaks shared the absorbance characteristics of native ADR (Fig. 2B). Peak c (Fig. 2A) co-eluted with native ADR and would be mistaken for the parent drug by a fixed-wavelength UV detector. All these products, which lacked visible absorbance and did not fluoresce, would not be identified by a fluorescence detector. Although HPLC coupled to fluorescence detection is commonly used to determine the purity of anthracyclines, it is not the most ideal technique due to an inability to detect heat and light degradation products.

Qualitative analysis of anthracyclines extracted from patient blood, urine, tissue and tumour specimens

ADR, DNR and 4'-DOX all exhibited identical UV—visible spectra (Fig. 3) with absorbance maxima determined to be 233, 253, 290, 478, 492 and 530 nm. These values agree well with the absorbance maxima of ADR obtained under stringent analytical conditions [20]. Anthracyclines have indicator-like properties [21] being orange at acidic pH, red at neutral pH and purple above pH 9. The values quoted above refer to acidic conditions, which were dictated by the pH of the mobile phases employed (pH 3.2). 4-DMDNR lacks an absorbance maximum at 233 nm and its spectrum is to be found in Fig. 8B.

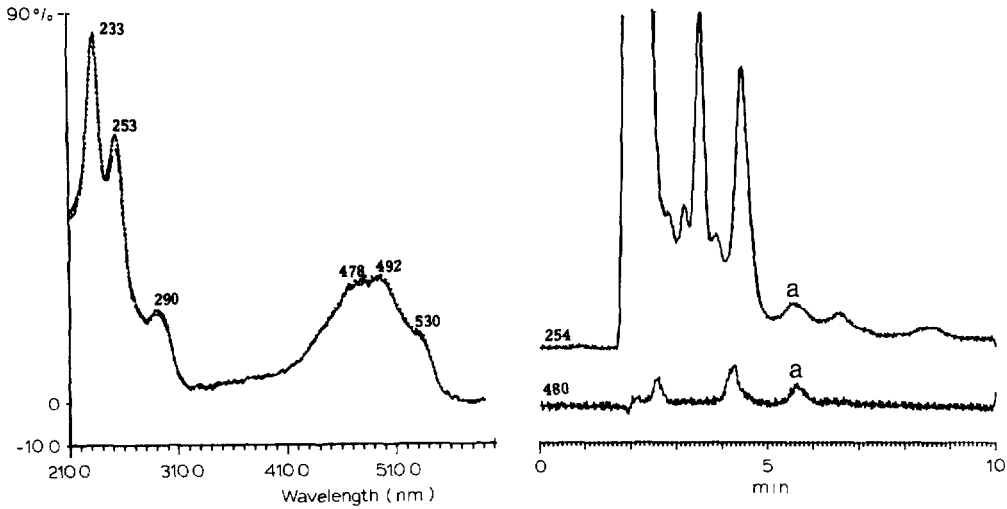


Fig. 3. UV—visible spectra of adriamycin (solid line), 4'-deoxydoxorubicin (dotted line) and daunorubicin (broken line) extracted from patient blood samples. The three spectra are superimposed. All were identical with all characteristic absorbance maxima intact indicating a pure compound in each case

Fig. 4. Chromatograms of 4-demethoxydaunorubicin extracted from blood taken 6 h after oral administration of 20 mg. Upper trace was monitored at 254 nm, full scale attenuation was 10 mA.U. Lower trace was monitored simultaneously at 480 nm, full scale attenuation was 2 mA.U. Peak a = 4-demethoxydaunorubicinol (20 ng/ml). Chromatographic conditions: mobile phase was 5 m*M* (final concentration) phosphoric acid in methanol, pH 3.2 (20:80) and the stationary phase was as in Fig. 2A. Elution was isocratic at a flow-rate of 2 ml/min.

Anthracyclines extracted from either plasma or serum retained their native UV—visible spectra (Fig. 3). A typical chromatogram of a plasma extract taken 6 h after drug administration and containing 2 ng/ml 4-DMDNR and 20 ng/ml 4-demethoxydaunorubicinol is displayed in Fig. 4. The lower trace was monitored at 480 nm and illustrates the profile a fluorescence detector would record. The upper trace was monitored at 254 nm and illustrates the number of non-anthracycline peaks actually present. Typical chromatograms of anthracyclines extracted from urine are displayed in Fig. 5 (4'-DOX) and Fig. 6A (4-DMDNR). In both cases the lower trace monitored at 480 nm (representing the fluorescence profile) indicated that the anthracyclines and their metabolites

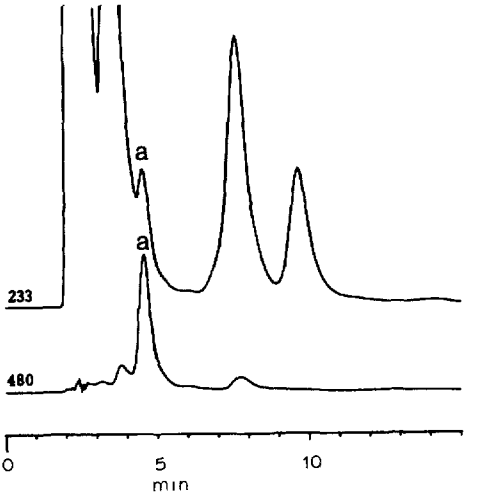


Fig. 5. Chromatograms of 4′-deoxydoxorubicin extracted from the urine of a patient treated with 42 mg. Upper trace was monitored at 233 nm, full scale attenuation was 100 mA.U. Lower trace was monitored simultaneously at 480 nm, full scale attenuation was 10 mA.U. Peak a = 4′-deoxydoxorubicin. Chromatographic conditions as in Fig. 2A.

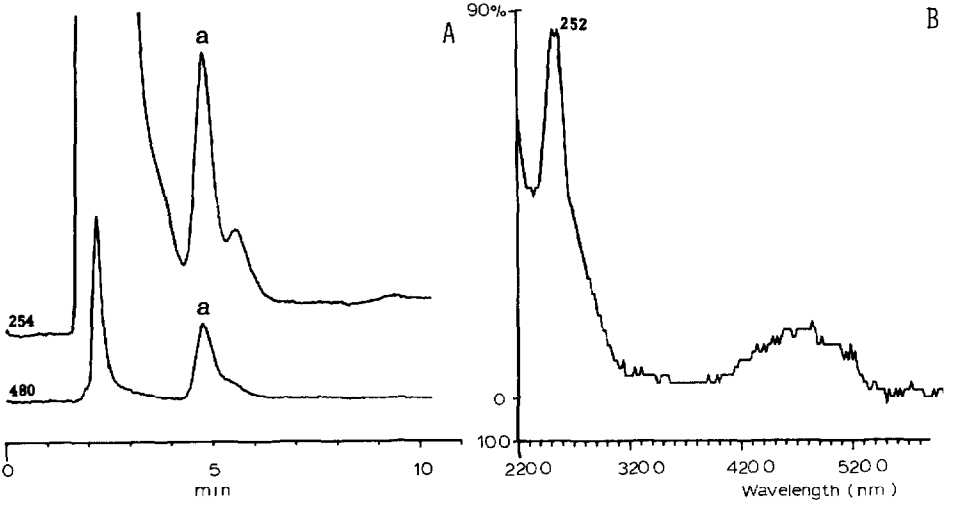


Fig. 6. (A) Chromatograms of 4-demethoxydaunorubicin extracted from the urine of a patient treated with 20 mg. Upper trace was monitored at 254 nm, full scale attenuation was 50 mA.U. Lower trace was monitored simultaneously at 480 nm, full scale attenuation was 10 mA.U Peaks: a = 4-demethoxydaunorubicinol. Chromatographic conditions as in Fig. 4. (B) UV—visible spectrum of 4-demethoxydaunorubicinol extracted from human urine (peak a). The UV maximum was shifted from 255 to 252 nm and considerable attenuation of visible absorbance was evidenced (compare with native spectrum, Fig. 8B).

were free from interfering peaks. However, the chromatograms monitored at the λ_{max} of each drug (upper traces) showed that co-eluting peaks were present. Spectral analysis of the anthracycline peaks confirmed the presence of impurities and the possibility of complexes. UV absorbance maxima were

shifted and attenuation in absorbance in the visible region was observed (Fig. 6B, compare with 8B). Quenching of anthracyclines would lead to a serious underestimation of their urinary excretion. A similar pattern was observed with liver and tumour biopsies containing ADR. Chromatograms monitored at 480 nm indicated pure specimens, whilst spectral analysis identified co-eluting impurities and also possibly complexes (Fig. 7).

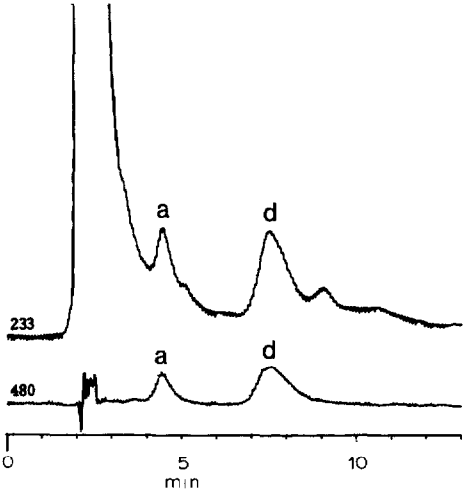


Fig. 7. Chromatograms of adriamycin extracted from liver biopsy resected 30 min after administration of 25 mg/m². Upper trace was monitored at 233 nm, full scale attenuation was 10 mA.U. Lower trace was monitored simultaneously at 480 nm, full scale attenuation was 5 mA.U. Peaks: a = adriamycin; d = daunorubicin (internal standard). Chromatographic conditions as in Fig. 2A.

It is, perhaps, not unreasonable to conclude that anthracyclines were present in urine and tissue samples as complexes. Anthracyclines are chemically reactive and bind avidly to protein and nucleic acids, both in vitro and in vivo, reversibly and covalently. ADR has been demonstrated to form spontaneous hydrophobic and ionic associations with amino acids, DNA base pairs [22], flavin nucleotides [23] and phospholipid components of biomembranes [24]. Under certain circumstances ADR will interact with itself, forming both dimers and polymers [25]. With the aid of high-speed spectrophotometric detection we have shown that anthracyclines in urine and tissue samples are likely to be either co-eluting with impurities or complexed to a degree.

Identification of chemically reduced anthracycline products

The main anthracycline metabolites (C_{13} alcohols, 7-deoxyaglycones and 7-hydroxyaglycones, Fig. 1B) are chemically modified at sites removed from the benzanthraquinone chromophore. All have UV—visible spectra identical or near identical to their parent compounds. They are also readily resolved by reversed-phase HPLC with fluorescence detection [15, 16]. One- and two-electron quinone reduction alters the electronic configuration of the chromophore nucleus to produce a series of reactive, semi-reactive and stable intermediates. These include the partially reduced semi-quinone free radical

(half-life less than 10^{-6} s [26]), fully reduced hydroquinone, a C_7 aglycone radical, a quinone methide aglycone and a 7-deoxyaglycone metabolite end product. All have their own unique absorbance characteristics; some very different from their parent compounds.

Aerobic chemical reduction of 4'-deoxydoxorubicin, adriamycin and 4-demethoxydaunorubicin

Fig. 8A is a chromatogram of the end product of aerobic reduction of 4-DMDNR. Three intact benzanthracene species were resolved, neither of

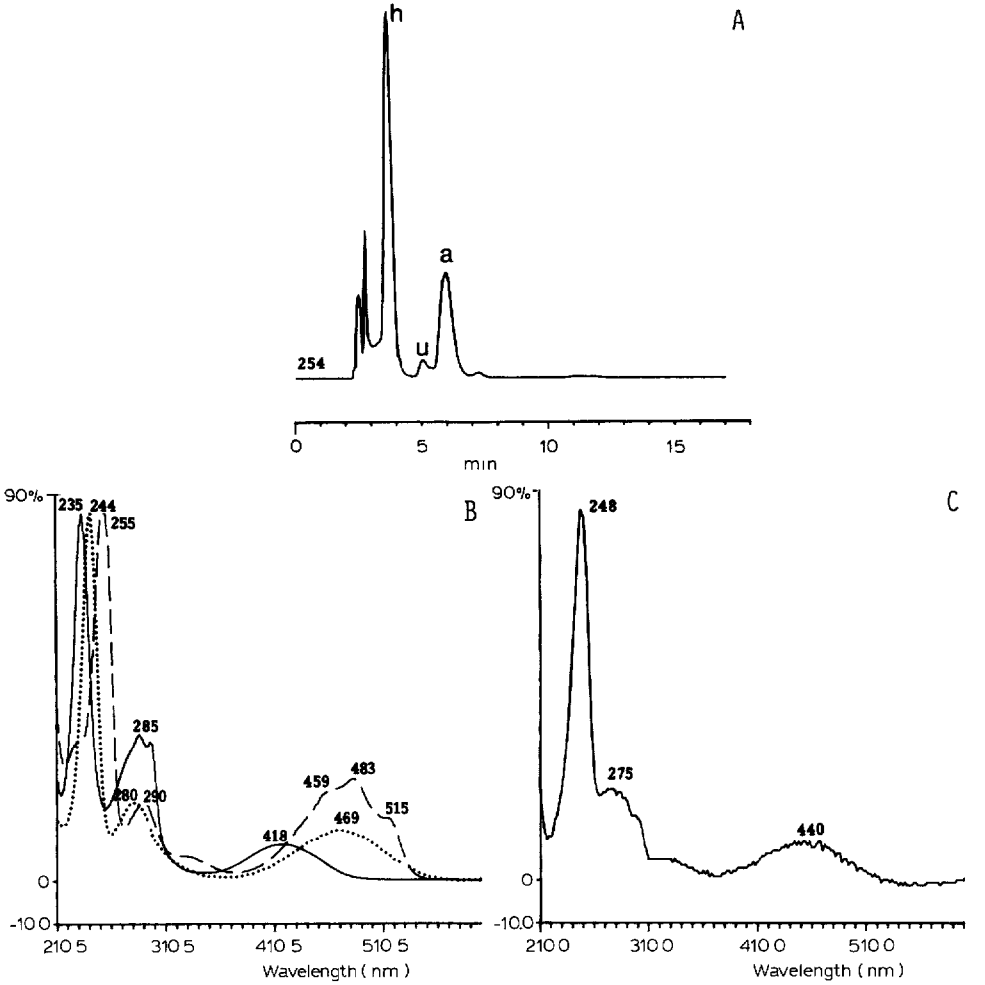


Fig. 8. (A) Chromatogram of the end product of aerobic reduction of 4-demethoxydaunorubicin with sodium borohydride. Peaks were monitored at 254 nm and full scale attenuation was 50 mA.U. Peaks: a = 4-demethoxydaunorubicinol; h = fully reduced hydroquinone; u = unidentified. Chromatographic conditions as in Fig. 4. (B) UV—visible spectra of peaks a, h and u from A. The spectrum of peak a (broken line) has the characteristic absorbance maxima of intact 4-demethoxydaunorubicinol. The spectrum of peak h (solid line) has the characteristic absorbance maximum (418 nm) of quinone-reduced anthracyclines. The spectrum of peak u (dotted line) has characteristics of both a and h. (C) UV—visible spectrum of fully reduced adriamycin and 4'-deoxydoxorubicin.

which co-eluted with the parent compound. Their UV—visible spectra are shown in Fig. 8B. Two of these peaks were identified from their spectra. Peak a was 4-demethoxydaunorubicinol (capacity factor, k' = 3.1) and peak h was fully reduced hydroquinone (k' = 1.5), a non-fluorescent compound with an absorbance maximum at 418 nm. Production of ADR hydroquinone by a pulse radiolysis technique yielded an end product with a visible absorbance maximum at 410 nm at pH 5 and 430 nm at pH 11 [21]. The third compound (peak u) was unidentified but its UV—visible spectrum was intermediate between the quinone and hydroquinone compounds (Fig. 8B). The UV—visible spectrum of fully reduced ADR and 4′-DOX is shown in Fig. 8C. These hydroquinones eluted after their parent quinone compounds (ADR, k' = 3.6; reduced ADR, k' = 4.1 and 4′-DOX, k' = 1.4; reduced 4′-DOX, k' = 2.0). However, the HPLC assays of ADR and 4′-DOX employed more polar mobile phases than 4-DMDNR [15, 16] and this may account for the difference in elution profiles. No trace of 7-deoxyaglycone metabolites was detected in any incubation.

Anaerobic chemical reduction of adriamycin in the presence of DNA

Anaerobic reduction of ADR produces a series of reactive aglycone intermediates postulated to bind covalently to proteins and nucleic acids [8, 19]. The reversible binding of ADR or DNR to DNA in vitro by intercalation results in a red shift in visible absorbance maximum from 495 to 506 nm and large hypochromic effects [27]. A typical chromatogram of the soluble end products of chemical reduction of ADR in the presence of DNA is shown in Fig. 9A. DNA and large polynucleotide fragments were retained on μBondapak guard columns. In another series of experiments the DNA was isolated and the nature of the ADR binding modes studied. The major intact anthracycline

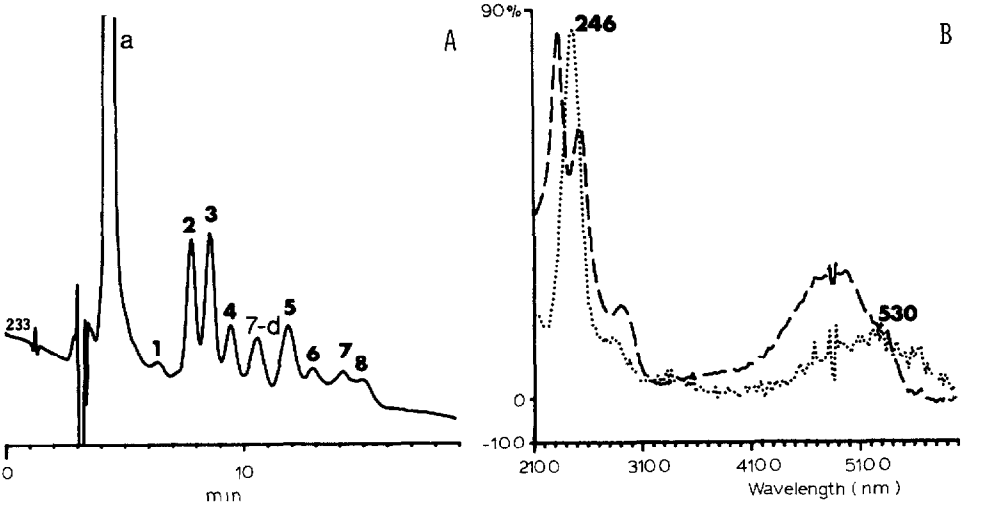


Fig. 9. (A) Chromatogram of the end product of anaerobic reduction of adriamycin with sodium borohydride in the presence of DNA. Peaks were monitored at 233 nm and full scale attenuation was 20 mA.U. Peaks: a = adriamycinol; 7-d = adriamycinol 7-deoxyaglycone; 1—8 = unidentified. Chromatographic conditions as in Fig. 2A. (B) UV—visible spectrum of peak a (adriamycinol, broken line) and peaks 1—8 (dotted line) from A. Peaks 1—8 all shared identical spectra.

species was adriamycinol (peak a, Fig. 9A); adriamycinol 7-deoxyaglycone was also present but no hydroquinone was detected. Eight other ADR-related peaks were resolved, all of which did not fluoresce when passed through a fluorescence detector connected in series to the diode array detector. All these peaks had their visible absorbance maximum shifted from 492 to 530 nm (Fig. 9B) and were coloured purple/blue. Whilst these peaks remain unidentified their purple colour is highly suggestive of the ADR bis(phenolate) ion. In the aerobic reductions of ADR (in absence of DNA) blue colouration appeared, but for only a fraction of a second before it was replaced by the yellow of the hydroquinone. In the anaerobic reductions (in the presence of DNA) the blue colouration was long lived, decaying only slowly over a period of 24–48 h. It is possible that the bis(phenolate) ion was stabilised by interacting with DNA through ionic bonds with the base pairs rich in nitrogen ready to act as an electron acceptor.

In conclusion, HPLC and high-speed spectrophotometric detection has resolved many important chemically formed anthracycline intermediates. Most of these would not have been identified by fluorescence or electrochemical detectors. A broad application for HPLC–multi-diode array detection is envisaged in the identification of biotransformed intermediates and complexes of anthracyclines in vivo.

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