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Method for the determination of 4-Demethoxydaunorubicin, its quinone and hydroquinone metabolites in human plasma and urine by high-performance liquid chromatography

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Summary. 4-Demethoxydaunorubicin (4-DMDNR) is a new orally active analogue of daunorubicin (DNR). We have developed a high-performance liquid chromatography (HPLC) method capable of separating and identifying 4-DMDNR, five possible fluorescent quinone metabolites and three possible non-fluorescent hydroquinone metabolites. Methods are described for high-yield synthesis of reference metabolites. The limit of detection of the fluorescence assay was less than 1 ng/ml after extraction of 1 ml plasma or urine with chloroform/propan-2-ol (2:1), with coefficients of variation in k' (HPLC column capacity factors) of less than 3% throughout the day. Efficiency of the extraction method described exceeded 80% in control experiments. Blood and urine samples were analysed from four cancer patients who had received 50 mg/m^2 orally as three divided doses every 8 h. A typical urinary profile of the drug and its metabolites was: parent drug, 13%; 4-demethoxydaunorubicinol (4-DMDNOL), 80%; 4-DMDNR 7-hydroxyaglycone, 4% and 4-DMDNOL 7-hydroxyaglycone, 3%. 4-DMDNOL was the major metabolite detected in plasma. A further metabolite identified as the 7-deoxyaglycone of 4-DMDNOL was detected in plasma of two patients at concentrations equal to or greater than the parent drug. In the other two patients no trace of the metabolite was detected.

Introduction

4-Demethoxydaunorubicin (4-DMDNR, Table 1) is a potent, more lipophilic analogue of the anti-cancer drug daunorubicin (DNR) which has recently completed a number of phase I and II clinical trials. 4-DMDNR differs structurally from DNR in the lack of a methoxy group on position C4 of ring D (Table 1). The drug is active against a number of solid tumours when administered orally to animals [8] and has activity against breast cancer when administered orally to patients [12]. It is more avidly taken up into isolated tumour cells growing in culture than DNR [17] and is retained in animal tissues for a longer period of time [2]. The increased cytotoxicity and anti-tumour activity of 4-DMDNR over DNR have been claimed to be due to the drug's lipophilicity and different in vivo disposition rather than a biochemical mechanism involving greater binding to DNA [7, 16].

After oral administration to cancer patients 4-DMDNR is extensively metabolised to the alcohol 4-demethoxydaunorubicinol (4-DMDNOL, Table 1) by a reaction which is catalysed for DNR by cytoplasmic NADPH-dependent aldo-keto reductases [10]. However, no other metabolic pathways have been described in man. A number of metabolic pathways are known to operate with DNR: hydrolytic cleavage of the C7 glycosidic linkage to produce 7-hydroxyaglycones (Table 1); quinone one and two electron reduction to produce 7-deoxyaglycones with the hydroquinone form as intermediate (Table 1); and conjugation with glucuronic acid and sulphate [18, 20].

4-DMDNR is one of only a few of the many anthracycline anti-cancer drugs that are active when given orally, and it is via this route that the drug is most likely to find a therapeutic role. Considering the potential for first-pass metabolism by the liver after oral administration, we have developed a rapid and sensitive HPLC method capable of separating and identifying the parent drug, 4-DMDNOL and several possible aglycone metabolites. Utilising two different detectors connected in series, a fluorescence monitor and a multi-diode array spectrophotometric detector (DAD), the method was able to distinguish between quinone and fully reduced hydroquinone forms of the drug and its metabolites. The method was applied to the analysis of blood and urine samples from four patients receiving 50 mg/m^2 orally in a phase II clinical trial for advanced small cell lung cancer.

Materials and methods

4-DMDNR-HCl, 4-DMDNOL-HCl and 4-demethoxydaunorubicin 7-deoxyaglycone (4-DMDNR-DONE, Table 1) were obtained from Farmitalia, Milan, Italy and were determined to be chromatographically pure. 4'-Deoxydoxorubicin (4'-DOX, the internal standard, Table 1) was also from Farmitalia. All chloroform and methanol used were HPLC reagent grade (Fisons Scientific Apparatus, Loughborough, UK); all other reagents, chemicals and solvents were of the highest grade available commercially. Water was de-ionised and bidistilled in a quartz glass still.

Synthesis of 7-hydroxyaglycones. 4-DMDNR or 4-DMDNOL was dissolved in 2 N HCl (5 mg in 10 ml) and incubated at 65°C for 1 h. The end product, 7-hydroxyaglycone, was insoluble in the acid and formed

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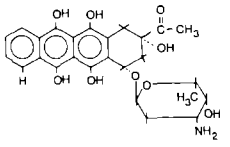
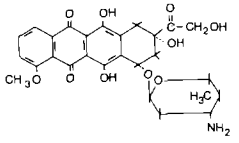
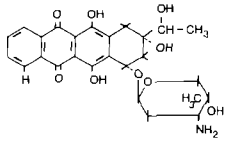
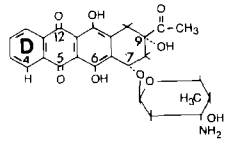
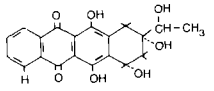
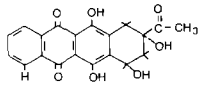
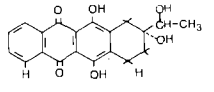
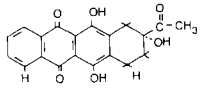
Table 1. 4-Demethoxydaunorubicin metabolites separated by HPLC

Structure	Compound	k' [a]	
		Quinone	Hydroquinone
	Fully reduced 4-Demethoxydaunorubicin (Hydroquinone) (hydro-4-DMDNR)		
	4'-Deoxydoxorubicin (internal standard) (4'-DOX)	2.8	
	4-Demethoxydaunorubicinol (4-DMDNOL)	4.2	2.2
	4-Demethoxydaunorubicin (Quinone) (4-DMDNR)	5.0	
	4-Demethoxydaunorubicinol 7-Hydroxyaglycone (4-DMDNOL-ONE)	6.3	3.4
	4-Demethoxydaunorubicin 7-Hydroxyaglycone (4-DMDNR-ONE)	7.9	
	4-Demethoxydaunorubicinol 7-Deoxyaglycone (4-DMDNOL-DONE)	12.6	6.7
	4-Demethoxydaunorubicin 7-Deoxyaglycone (4-DMDNR-DONE)	15.0	

[a] HPLC column capacity factors, k'

a red precipitate. The insoluble aglycone was washed three times with water to remove excess acid and was stored in crystalline form prior to analysis. The above conditions resulted in >99% hydrolysis, with no other intact benzanthracene products formed.

Synthesis of 7-deoxyaglycones. 7-Deoxyaglycones were synthesised by catalytic hydrogenation at standard temperature and pressure using a poisoned palladium/barium carbonate catalyst (5:95), 50 mg of which was added to 10 mg 4-DMDNR or 4-DMDNOL in 10 ml methanol. The mixture was shaken with hydrogen gas for 12 h. The end product was centrifuged, the methanol was separated and evaporated to dryness, and the 7-deoxyaglycone was redissolved in chloroform. The above conditions resulted in >90% conversion to aglycone, with no other benzanthracene products formed.

Synthesis of hydroquinones. 4-DMDNR or 4-DMDNR 7-hydroxyaglycone (4-DMDNR-ONE, Table 1) or 4-DMDNR 7-deoxyaglycone (4-DMDNR-DONE, Table 1) 2 mg was dissolved in 10 ml ethanol. At these concentrations, the two aglycones were not readily soluble in ethanol and were dissolved by heating at 70°C for 3 min. Addition of 200 µl of a freshly made aqueous solution of 3.8% sodium borohydride (38 mg/ml) followed, and the reduction was allowed to proceed for 5 min [19]. Addition of 1 drop of glacial acetic acid terminated the reduction. Hydroquinones were isolated by evaporation of the reaction mixture to dryness with nitrogen gas. Heating was avoided because it led to significant re-oxidation of the reduced quinone. Quinone and hydroquinone forms of anthracyclines can be separated by TLC [3]. The above conditions resulted in >97% quinone and carbonyl reduction (see Table 1), with no other benzanthracene end products formed.

Characterisation of synthesised reference metabolites. The quinone metabolites were characterised by UV-visible spectrophotometry (Gilford model 250 spectrophotometer), fluorescence spectrophotometry (Shimadzu fluorescence spectrophotometer) and mass spectrometry (Kratos MS 902S and DS 55C data system). Hydroquinone metabolites were characterised by UV-visible spectrophotometry and by their ability to be completely reoxidised back to the quinone form by bubbling with air for 24 h at STP in the dark. Fluorescence and UV-visible scans were performed on acidified methanolic solutions (pH 3) and direct probe injection mass spectrometry with methanolic solutions. All quinone metabolites shared identical UV-visible spectra with absorption maxima at 255, 290, 459, 483 and 515 nm (Fig. 1) and nearly identical fluorescence spectra with an excitation maximum at 488 nm and an emission maximum at 546 nm. The mass spectrum of 4-DMDNR-ONE yielded a molecular ion m/z 368 and a recognisable frag-

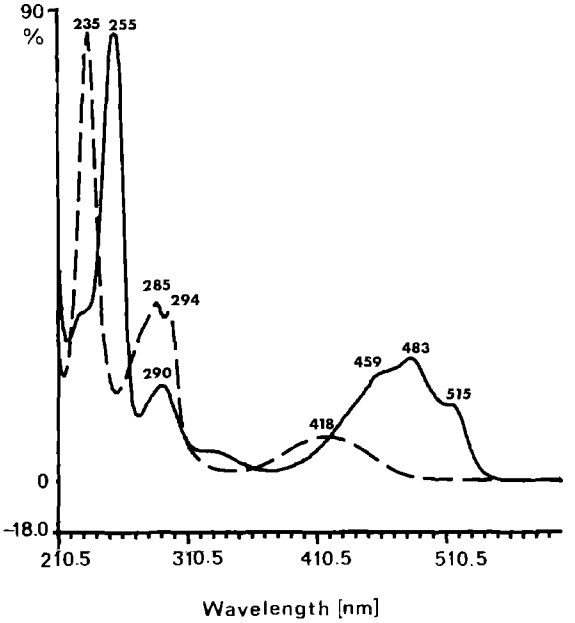


Fig. 1. UV-visible absorption spectra of the quinone *(solid line)* and hydroquinone *(broken line)* forms of 4-demethoxydaunorubicin and reference metabolites. Reference metabolites shared identical spectra to the parent drug

mentation pattern: m/z 332 (M-2H_2O) and m/z 307 (M-H_2O,-$COCH_3$). The mass spectrum of 4-DMDNR-DONE also yielded a molecular ion, m/z 352 with a similar fragmentation pattern: m/z 316 (M-2H_2O) and m/z 291 (M-H_2O,-$COCH_3$). All hydroquinone metabolites shared identical UV-visible spectra with absorption maxima at 235, 285, 294, and 418 nm but did not fluoresce (Fig. 1).

High-performance liquid chromatography. The liquid chromatograph used throughout was the same as previously described [5]. The injection port was equipped with a 20-µl injection loop. The chromatograph was connected in series to a Gilson Spectro-glo filter fluorimeter with narrow band interference filters at 480 nm for excitation and 560 nm for emission (Gilson, Villiers-le-Bel, France) and a Hewlett-Packard Model 1040A multidiode array high-speed scanning spectrophotometric detector (DAD) [4], with a 4.5 µl quartz micro flow cell, 6-mm path length (Hewlett-Packard, Manchester, UK).

The separation was achieved with 25 cm × 4.6 mm I. D. stainless steel columns packed with µ-Bondapak C18 (10-µm particles, Waters Associates, Northwich, UK) according to our published method [3]. The mobile phase consisted of 33% 5-m*M* phosphoric acid (final concentration) in 67% methanol, pH 3.2. eluting isocratically at a flow rate of 2 ml/min.

Quantitation and qualitative analysis of reference metabolite purity. The output from the detectors was directed to a Shimadzu CR-1B computing integrator. Calibration was by a two-point external standard method with ten replicate methanolic solutions containing, at concentrations of 20 ng/ml or 1 µg/ml, mixtures of 4-DMDNR, 4-DMDNOL, 4'-DOX, the four quinone aglycones and the three hydroquinones (Table 1). Above 10 µg/ml solubility problems were encountered with 7-deoxyaglycones. The DAD was employed to verify the identity of reference metabolites by UV-visible spectroscopy. Each synthesised reference metabolite, quinone and hydroquinone form was determined to be spectrophotometrically pure and eluting as a single chromatographic species with UV-visible spectra identical to each compound in solution (Fig. 1).

Extraction procedure from plasma or urine. 4-DMDNR and reference metabolites were extracted from plasma or urine with 5 vol of chloroform/propan-2-ol (2:1) according to the rapid method described for Adriamycin and its metabolites in a previous report [5]. 4'-DOX was the internal standard and 25 ng was normally added to patient plasma samples before extraction.

Patient samples. Blood and urine samples were collected from four female patients, aged 55, 57, 58 and 64 years. All had normal liver and kidney function, normal haemoglobin levels and normal white blood cell count, and none was a heavy drinker of alcohol. Each patient received a total dose of 50 mg/m^2 4-DMDNR, administered orally as three equal divided doses every 8 h as part of a phase II clinical trial for extensive small cell lung cancer. After blood sampling, plasma was separated and stored at −20°C in plain glass tubes. Plasma samples were transferred from clinic to laboratory in ice packs without thawing and stored again at −20°C prior to analysis. Urine collections were handled in a similar way to plasma. All samples were thawed at room temperature and immediately analysed.

Results and discussion

High-performance liquid chromatography

We have developed an HPLC method capable of separating and identifying 4-DMDNR, five possible quinone me-

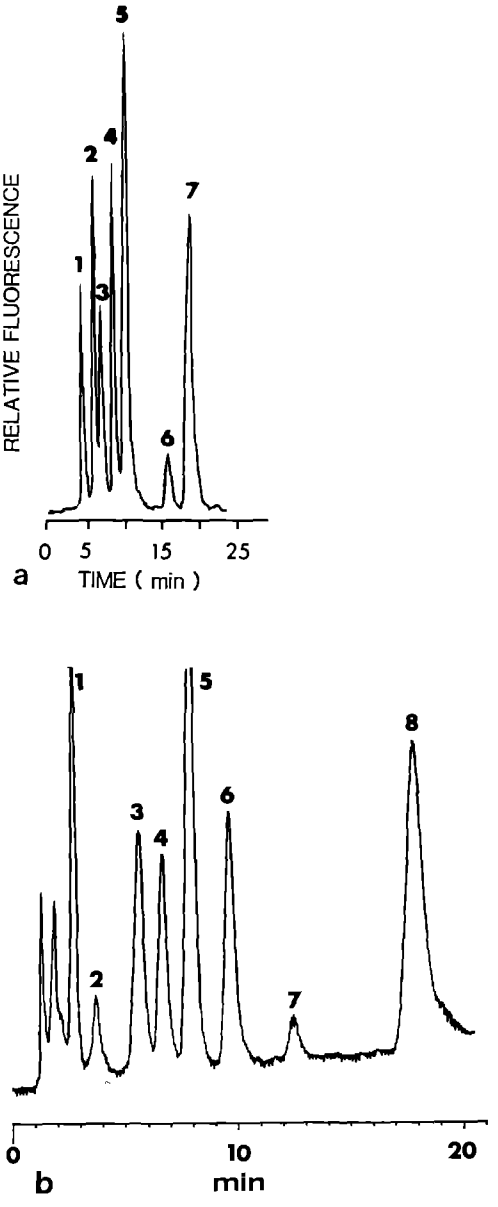


Fig. 2. a Separation of quinone forms of 4-demethoxydaunorubicin and reference metabolites by HPLC with fluorescence detection. *Peaks* are: *1*, 4'-DOX; *2*, 4-DMDNOL; *3*, 4-DMDNR; *4*, 4-DMDNOL-ONE; *5*, 4-DMDNR-ONE; *6*, 4-DMDNOL-DONE; *7*, 4-DMDNR-DONE. **b** Separation of quinone and hydroquinone forms of 4-demethoxydaunorubicin and reference metabolites by HPLC with diode array spectrophotometric detection. *Peaks* were monitored at 235 nm and full-scale attenuation was 5 milli-AU. *Peaks* are: *1*, hydro-4-DMDNOL; *2*, 4'-DOX; *3*, 4-DMDNOL; *4*, 4-DMDNR; *5*, hydro-4-DMDNOL-ONE; *6*, hydro-4-DMDNOL-DONE; *7*, 4-DMDNOL-DONE; *8*, 4-DMDNR-DONE. Structures are given in Table 1

tabolites and three possible hydroquinone metabolites. Column capacity factors (k') are contained in Table 1, along with chemical structure, trivial name and abbreviated name. The limit of detection of the fluorescence assay at a 3 : 1 ratio of peak height to baseline noise height was: 0.25 ng injected onto the column for 4-DMDNR, 4-DMDNOL, 4-DMDNR-ONE and 4-DMDNOL-ONE and 0.5 ng injected onto the column for 4-DMDNR-DONE and 4-DMDNOL-DONE. After extraction from 1 ml plasma, the limit of detection was 0.5 ng/ml for 4-DMDNR, 4-DMDNOL, 4-DMDNR-ONE and 4-DMDNOL-ONE and 1 ng/ml for the two 7-deoxyaglycones. Over the concentration range 5 ng/ml to 10 μg/ml fluorescence calibration curves were linear. Regression correlation coefficients *(r)* were 0.9995 for 4-DMDNOL, 0.9993 for 4-DMDNR, 0.9988 for 4-DMDNOL-ONE, 0.9982 for 4-DMDNR-ONE, 0.9922 for 4-DMDNOL-DONE and 0.986 for 4-DMDNR-DONE from 20-point calibration curves. Hydroquinones were quantitated by UV absorbance at 235 nm (their λmax). At an attenuation of 1.0 milli-AU the limit of detection in plasma was 10 ng/ml for hydro-4-DMDNOL, 20 ng/ml for hydro-4-DMDNOL-ONE and 20 ng/ml for hydro-4-DMDNOL-DONE. The separation was reproducible from column to column. Capacity factors (Table 1) varied by less than 3% (coefficient of variation) for all quinone and hydroquinone reference metabolites over an 8 h period, after equilibration with mobile phase for 1 h at the start of the day. A typical fluoresence chromatogram of the separation of reference quinone metabolites is shown in Fig. 2a and a typical UV chromatogram of the separation of hydroquinone and quinone reference metabolites is shown in Fig. 2b. No endogenous fluorescent components extracted from plasma interfered with the identification of 4-DMDNR and its metabolites (Fig. 3a). Whilst the separation achieved relatively large values of k, the latest eluting peak (4-DMDNR-DONE, Table 1) had a retention time (E_r) of 18 min and an analysis could be completed in under 20 min. The t_r of a non-retained compound was 1.1 min.

Extraction procedure

Percentage recoveries for 20 ng extracted from 1 ml of blood bank plasma were high, with low standard deviation for each reference standard (% ± SD). Extraction efficiency was 93 ± 2.1 for 4-DMDNOL, 95 ± 3.1 for hydro-4-DMDNOL, 94 ± 4.2 for 4-DMDNR, 92 ± 2.3 for 4-DMDNOL-ONE, 94 ± 3.6 for hydro-4-DMDNOL-ONE, 90 ± 4.5 for 4-DMDNR-ONE, 86 ± 3.7 for 4-DMDNOL-DONE, 90 ± 2.8 for hydro-4-DMDNOL-DONE and 80 ± 3.6 for 4-DMDNR-DONE. The rapid extraction method recovers the internal standard 4'-DOX with similar high efficiency (81.4 ± 2.6).

To date, only HPLC methods to separate 4-DMDNR and 4-DMDNOL have been reported in the literature [1, 2, 13–15]. These methods have either employed isocratic elution with mobile phases consisting of 20%–45% acetonitrile in acidified aqueous buffer or gradient elution with phosphate buffer, pH 3.0 and acetonitrile : phosphate buffer, pH 3.0. In the first case 4-DMDNR and 4-DMDNOL were well resolved using low flow rates (0.4 ml/min). However, analysis times were prolonged (4-DMDNR, t_r 24 min), peaks were broadened, sensitivity was reduced, and aglycones would be retained for some hours. In the case of gradient elution, 4-DMDNR and 4-DMDNOL were less well resolved but analysis time was still prolonged (4-DMDNR, t_r 15 min approx.) and aglycones would still be retained for some hours. The *n*-butanol/Tris-HCl buffer partition coefficient of 4-DMDNR is almost 5 times greater than that of Adriamycin and twice

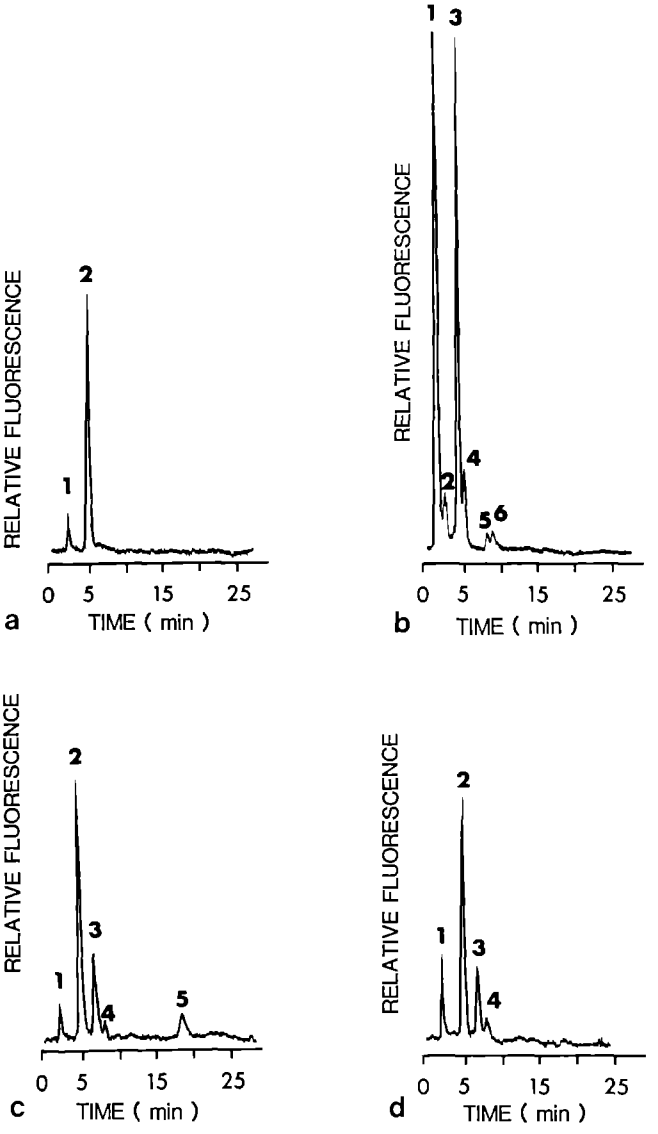


Fig. 3. **a** Chromatogram with fluorescence detection of pre-treatment plasma extracted by the rapid method with chloroform: propan-2-ol (2 : 1). *Peaks* are: *1*, endogenous; *2*, 4'-DOX (internal standard). No endogenous fluorescent peaks were extracted which interfered with the identification of 4-demethoxydaunorubicin and quinone reference metabolites. **b** Chromatogram with fluorescence detection showing a typical profile of metabolites identified in patient urine. Urine was from the first 6 h collection after the last oral divided dose. *Peaks* are: *1* and *2*, unidentified endogenous components; *3*, 4-DMDNOL (41 ng/ml); *4*, 4-DMDNR (9.8 ng/ml); *5*, 4-DMDNOL-ONE (1.4 ng/ml); *6*, 4-DMDNR-ONE (2.1 ng/ml). **c** Chromatogram with fluorescence detection of patient plasma taken 6 h after the last oral divided dose. *Peaks* are: *1*, endogenous components; *2*, 4'-DOX (internal standard); *3*, 4-DMDNOL (22 ng/ml); *4*, 4-DMDNR (3 ng/ml); *5*, 4-DMDNOL-DONE (7 ng/ml). **d** Chromatogram with fluorescence detection of plasma taken 6 h after the last oral divided dose. *Peaks* are: *1*, endogenous components; *2*, 4'-DOX (internal standard); *3*, 4-DMDNOL (13 ng/ml); *4*, 4-DMDNR (5 ng/ml)

that of DNR [9]. 4-DMDNR is considerably less water-soluble than Adriamycin, and while acetonitrile is a suitable organic modifier in Adriamycin separations it is probably not ideal for 4-DMDNR. We used a mobile phase that contained a high percentage of methanol, and this enabled a quick and efficient separation of 4-DMDNR, 4-DMDNOL and aglycones with no peak tailing and high sensitivity.

Analysis of patient samples

7-Hydroxyaglycones were not detected in any plasma sample from all patients studied but were minor constituents present in urine. A fluorescence chromatogram of a urine extract is shown in Fig. 3b. A typical urinary profile of metabolites was: parent drug, 13%; 4-DMDNOL, 80%; 4-DMDNR-ONE, 4% and 4-DMDNOL-ONE, 3%. The hydroxyaglycone of the parent drug was always the most abundant urinary aglycone. In some urine samples no 4-DMDNOL-ONE was detected.

4-DMDNR concentrations in plasma were low, rarely exceeding 10 ng/ml, and were similar in all four patients (peak concentration [PC], 6-12ng/ml; half-life [$t_{1/2}$], 4–11 h). The parent drug was usually no longer detectable in plasma in 24 h after the last divided dose. 4-DMDNOL concentrations in plasma, like urine, were several times higher than the parent drug (PC, 18–47 ng/ml; $t_{1/2}$ 25–121 h). Another metabolite was clearly detectable in plasma: it chromatographed with t_r and k' identical to those for the reference metabolite of 4-DMDNOL-DONE (Table 1) and had a UV-visible spectrum consistent with the quinone form of 4-DMDNR (see Fig. 1). The metabolite was only detected in plasma samples of two of the four patients (Fig. 3c: PC, 7–8 ng/ml; $t_{1/2}$, 4–8 h). In the other two patients no trace was detected at any time point (Fig. 3d).

7-Deoxyaglycone metabolites of anthracyclines are end products of important biotransformations which result in the formation of semiquinone-free radicals, hydroquinones and reactive radical aglycone intermediates. All these species, if generated in vivo, could theoretically participate in and alter both the therapeutic and the toxicological properties of the drugs.

The presence of a 7-deoxyaglycone of 4-DMDNR in the plasma of two cancer patients raises the possibility that this drug undergoes quinone reduction and is subsequently converted to reactive intermediates in man. The observation that some patients produced the 7-deoxyaglycone whilst others did not may have pharmacological importance. We have recently reported a similar phenomenon for Adriamycin in a much larger cohort of patients [6].

In conclusion, we present evidence for more extensive biotransformation of 4-DMDNR than has originally been reported [1, 11]. A marked inter-patient variation in conversion of 4-DMDNR to a plasma metabolite identified as 4-DMDNOL-DONE was observed. These metabolic differences may have clinical relevance for both the therapeutic and toxicological properties of the drug.

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