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METHOD FOR THE DETERMINATION OF 4′-DEOXYDOXORUBICIN, 4′-DEOXYDOXORUBICINOL AND THEIR 7-DEOXYAGLYCONES IN HUMAN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

4′-Deoxydoxorubicin (4′-DOX) is a new and structurally similar analogue of the anticancer drug adriamycin (ADR). Based on known pathways of metabolism of ADR a high-performance liquid chromatographic method for the separation and identification of 4′-DOX and five possible metabolites was developed. Sensitivity for serum is 10 ng/ml for 4′-DOX and its alcoholic product 4′-deoxydoxorubicinol (4′-DOL) and 2 ng/ml for four of its aglycone products with coefficients of variation in k' of less than 5% throughout the day. An extraction step with better than 80% recovery of 4′-DOX and five reference metabolites from serum is described. Analysis of patient sera identified two metabolite peaks. These co-eluted with the reference metabolites of 4′-DOL and the 7-deoxyaglycone of 4′-DOX. Pharmacokinetics of the parent drug followed a two-compartment model. Both the metabolites were produced quickly and disappeared quickly.

INTRODUCTION

An irreversible toxicity to the heart with a high risk of mortality limits the clinical administration of the therapeutically active anthracycline anticancer drug adriamycin (ADR) to a cumulative dose of 550 mg/m^2 [1]. Many analogues have been synthesised in the hope that one will be equally or more cytotoxic than the parent drug but without its cardiotoxicity [2, 3]. 4′-Deoxydoxorubicin (4′-DOX) is one of the most promising newly synthesised anthracyclines. Structurally it is identical to ADR differing only by the absence of an oxygen atom at position 4 on the daunosamine sugar group (Fig. 1). Like ADR it has a broad spectrum of anti-tumour activity but is thought to be more effective [4, 5]. Of all the new anthracycline analogues tested 4′-DOX appeared to be the least cardiotoxic [6]. However, its systemic toxicity is greater than

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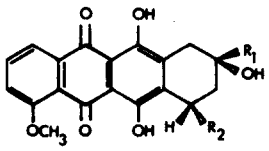


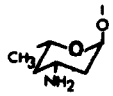
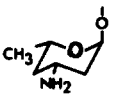
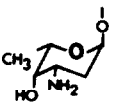
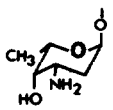
COMPOUND	R_1	R_2
4'- Deoxydoxorubicin (4'- DOX)	$-C(=O)-CH_2OH$	
4'- Deoxydoxorubicinol (4'- DOL)	$-CH(OH)-CH_2OH$	
Adriamycin (ADR)	$-C(=O)-CH_2OH$	
Adriamycinol (AOL)	$-CH(OH)-CH_2OH$	
4'- Deoxydoxorubicin aglycone (4'-DOX- one), also Adriamycin aglycone (ADR-one)	$-C(=O)-CH_2OH$	OH
4'- Deoxydoxorubicinol aglycone (4'- DOL-one), also Adriamycinol aglycone (AOL-one)	$-CH(OH)-CH_2OH$	OH
4'- Deoxydoxorubicin 7 - deoxyaglycone (4'-DOX-done), also Adriamycin 7 - deoxyaglycone (ADR-done)	$-C(=O)-CH_2OH$	H
4'- Deoxydoxorubicinol 7-deoxyaglycone (4'- DOL-done), also Adriamycinol 7 - deoxyaglycone (AOL-done)	$-CH(OH)-CH_2OH$	H

Fig. 1. The structure of 4′-deoxydoxorubicin and five of its possible metabolites plus the structure of adriamycin and its metabolites.

ADR and its administration is limited to approximately half the therapeutic dose of ADR [5, 7, 8]. Preliminary reports indicate that 4′-DOX is also non-cardiotoxic in man [9, 10]. Although, in one study a patient developed clinical signs of congestive heart failure after only three courses of 30 mg/m^2 4′-DOX [10].

Two major pathways of metabolism have been described for ADR [11—14]: reduction of the carbonyl group on the alkyl side-chain by a cytoplasmic aldo—keto reductase enzyme to yield the alcohol adriamycinol (AOL, Fig. 1) and microsomal reductive glycosidic cleavage of ADR and AOL to 7-deoxyaglycones

(Fig. 1). It is not unreasonable to expect 4′-DOX also to be metabolised in a similar fashion because of its close structural similarity to ADR.

In this paper a high-performance liquid chromatographic (HPLC) method is presented for determining 4′-DOX, 4′-deoxydoxorubicinol (4′-DOL) and their 7-deoxyaglycones in the serum of cancer patients.

MATERIALS AND METHODS

Apparatus

HPLC was performed throughout using an Altex Model 110A pump and an Altex Model 210 injection port with a 20-μl injection loop (Beckman-RIIC, High Wycombe, U.K.); a Gilson Spectro-Glo filter fluorimeter with narrow-band interference filters at 480 nm (excitation) and 560 nm (emission) and a 10-μl quartz micro flow cell (Gilson, Villiers-le-Bel, France); a Shimadzu CR-1B computing integrator (supplied by Scotlab Instrument Sales, Bellshill, U.K.) and 250 mm × 4.6 mm I.D. stainless-steel columns packed with μBondapak C_{18} (10 μm particle size, Waters, Northwich, U.K.) using a Shandon HPLC column packer (Shandon, Runcorn, U.K.). Columns were packed according to the following method: 4 g of μBondapak in a propan-2-ol slurry was packed into a column under a pressure of 480 bars in an upward direction with 100 ml propan-2-ol, the column was inverted and packing was continued in a downward direction with 50 ml propan-2-ol and finally conditioned in a downward direction with 50 ml methanol and 50 ml of the mobile phase described in this paper.

Reagents and reference compounds

All methanol, propan-2-ol and chloroform were HPLC-reagent grade (Fisons Scientific Apparatus, Loughborough, U.K.). Orthophosphoric acid and all other solvents and chemicals were of analytical reagent grade (AnalaR, BDH, Poole, U.K.). Water was deionised and double-distilled in a quartz glass still. Pure adriamycin·HCl and adriamycinol·HCl were a gift from Dr. S. Penco (Farmitalia, Milan, Italy). 4′-Deoxydoxorubicin containing 5 mg lactose per mg of drug was from Farmitalia. Daunorubicin·HCl (DNR), the internal standard, was from May and Baker (Dagenham, U.K.). 4′-DOL was synthesised from 4′-DOX by reduction with sodium borohydride in a reaction analogous to the reduction of ADR to AOL [15]. Quinone groups reduced during the reaction were regenerated by bubbling air through the reaction mixture for 2 h. Purity of the alcohol was assessed by thin-layer chromatography (TLC) using 20 × 10 cm glass plates coated with a 250-μm layer of silica gel G (Analtech uniplates, Scotlab Instrument Sales) and three different ascending solvent systems (S). S1 was chloroform—methanol—water (80:20:3); S2 chloroform—methanol—acetic acid—water (80:20:14:6) and S3 ethyl acetate—ethanol—acetic acid—water (80:10:5:5) [15]. Spots were visualised under UV light at 254 nm. The reaction mixture still contained a significant quantity of the reduced quinone compound (a yellow non-fluorescent substance) but was otherwise pure. 4′-DOL, a red substance, was separated from the reduced quinone compound and purified by preparative TLC using 20 × 20 cm glass plates coated with a 1-mm layer of silica gel G (Analtech uniplates) after running the plate in S3 for 10 cm, drying and then running in S2 for 18 cm. The alcohol was eluted from

the silica gel using propan-2-ol and spectro-photometric analysis confirmed the red compound had a UV—visible spectrum identical to 4′-DOX. Four aglycones were used as reference compounds. The aglycone group of ADR and 4′-DOX is identical (Fig. 1), consequently their aglycone metabolites would be identical as well (see Fig. 1). The aglycones of 4′-DOX (4′-DOX-one) and 4′-DOL (4′-DOL-one) and the 7-deoxyaglycones of 4′-DOX (4′-DOX-done) and 4′-DOL (4′-DOL-done) were synthesised and characterised as previously described [16].

Calibration

Integrator calibration was performed by an external standard method. Five stock solutions containing mixtures of 100 μg/ml ADR, AOL, 4′-DOX, 4′-DOL, DNR, 4′-DOX-one, 4′-DOL-one and 10 μg/ml 4′-DOX-done and 4′-DOL-done were prepared in methanol and diluted in methanol. For the calibration 20 ng of the two 7-deoxyaglycones and 100 ng of all the other reference compounds were applied on to the column. Standard solutions were stored in PTFE-lined capped bottles at −20°C and were made up fresh every two months. Detector response was linear over the range 2—2000 ng injected on column for all nine reference compounds ($r = 0.996$). The limit of detection was set at the 3:1 signal-to-noise ratio.

Chromatographic conditions

The mobile phase consisted of 5 m*M* (final concentration) orthophosphoric acid—propan-2-ol, pH 3.2 (74:26). Elution was isocratic at a flow-rate of 1.2 ml/min. Mobile phase was filtered (0.22 μm filter pore size, Waters) and sonicated at 12 μm for 15 min before use (MSE Instruments, Crawley, U.K.). The column was maintained at ambient room temperature.

Rapid extraction from serum

Blood samples were collected from patients receiving 40 mg/m^2 ADR and 30 mg/m^2 4′-DOX and were allowed to clot in plain glass tubes. Sera were then separated by centrifugation at 1000 *g* for 10 min and stored at −20°C in plain PTFE-lined screw capped tubes. The technique used to extract 4′-DOX and its metabolites was identical to that used to extract ADR and its metabolites and has already been described in detail [16]. Essentially, the method involved direct extraction from 1 or 2 ml serum with 5 vols. chloroform—propan-2-ol (2:1) for 30 min with vortexing (100 ng DNR was added as an internal standard). After centrifugation at 2000 *g* for 15 min at 4°C, the upper aqueous phase was discarded and the lower organic phase transferred to a clean tube and evaporated to dryness at 40°C. The extract was redissolved in either 40, 50 or 100 μl of methanol and 20 μl was applied on to the HPLC column.

RESULTS

High-performance liquid chromatography

In Fig. 2 is an example of the separation of 4′-DOX and its reference metabolites (Fig. 2A) and ADR and its metabolites (Fig. 2B). Retention times (t_R) and capacity factors (k') are shown in Table I. In control experiments over an 8-hr period at 27 ± 0.5°C the coefficient of variation (C.V.) in k' was less than

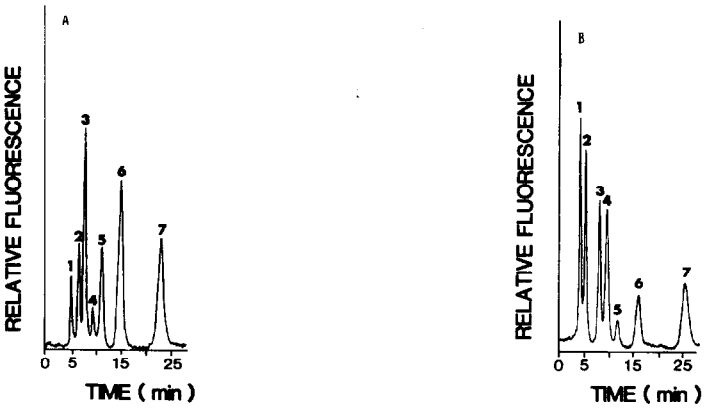


Fig. 2. Chromatographic separation of reference compound mixtures. (A) 4′-DOX and five of its possible metabolites. Peaks are identified as: 1 = 4′-DOL; 2 = 4′-DOX; 3 = 4′-DOL-one; 4 = DNR; 5 = 4′-DOX-one; 6 = 4′-DOL-done; 7 = 4′-DOX-done. (B) ADR and metabolites. Peaks are identified as: 1 = AOL; 2 = ADR; 3 = AOL-one; 4 = DNR; 5 = ADR-one; 6 = AOL-done; 7 = ADR-done. Chromatographic conditions: mobile phase was 5 m*M* ortho-phosphoric acid—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 and detection was by fluorescence at 480 nm (excitation) and 560 nm (emission). Chemical structures of all reference compounds are to be found in Fig. 1.

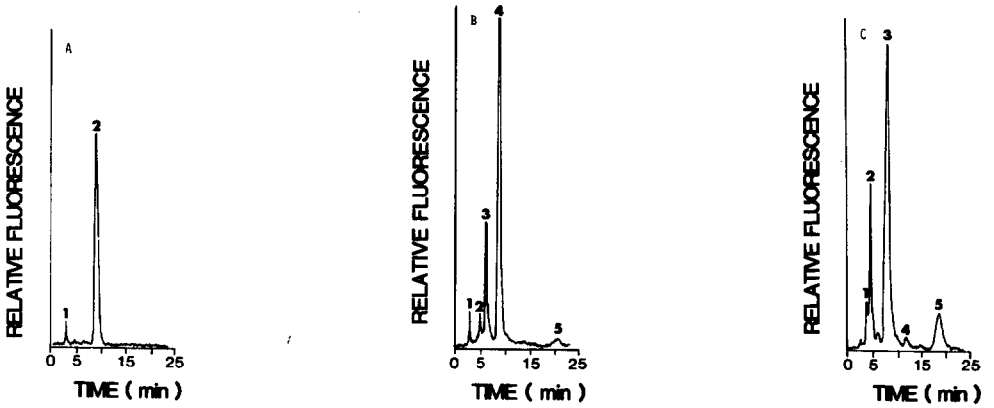


Fig. 3. Chromatographic analysis of serum from cancer patients. (A) Serum extract of blood taken prior to drug administration. Peaks are identified as: 1 = serum peaks; 2 = DNR (50 ng/ml). (B) Serum extract of blood taken 20 min after administration of 30 mg/m^2 4′-DOX as an i.v. bolus injection. Peaks are identified as: 1 = serum peaks; 2 = 4′-DOL (6.7 ng/ml); 3 = 4′-DOX (173 ng/ml); 4 = DNR; 5 = 4′-DOX-done (3.4 ng/ml). (C) Serum extract of blood taken 20 min after administration of 40 mg/m^2 ADR as an i.v. bolus injection. Peaks are identified as 1 = AOL (31 ng/ml); 2 = ADR (138 ng/ml); 3 = DNR; 4 = AOL-done (8 ng/ml); 5 = ADR-done (49 ng/ml). Chromatographic conditions as in Fig. 2 except for C where the flow-rate was 1.3 ml/min.

5% for all of the nine reference compounds (for example, C.V. for 4′-DOX was 2.0%, for 4′-DOL 2.1% and 4′-DOX-done 2.7%). The limit of detection as an amount injected on to the column was 5 ng for 4′-DOX and 4′-DOL and 1 ng for the four 4′-DOX aglycones. Limit of detection in serum after extraction of 1 ml was 10 ng/ml for 4′-DOX and 4′-DOL and 2 ng/ml for the four 4′-DOX

TABLE I

SEPARATION OF 4'-DEOXYDOXORUBICIN AND ITS REFERENCE METABOLITES BY ISOCRATIC REVERSED-PHASE HPLC USING μBONDAPAK C_{18}

Compound*	t_R(min)	k'	Compound*	t_R(min)	k'
4'-DOX	5.9	1.4	ADR	5.4	1.2
4'-DOL	4.8	0.9	AOL	3.9	0.6
4'-DOL-one	7.4	2.0	DNR	8.7	2.5
4'-DOX-one	10.6	3.2			
4'-DOL-done	13.8	4.5			
4'-DOX-done	21.1	7.4			

*Chemical structures to be found in Fig. 1.

aglycones. Extraction from serum did not introduce peaks which interfered with the identification of 4'-DOX and its reference metabolites (Fig. 3A).

Serum extraction

Extraction efficiency from blood bank serum of 100 ng 4'-DOX was 81.4 ± 2.6% S.D. (n = 10) and 100 ng of 4'-DOL was 80.6 ± 4.3% S.D. (n = 10). The rapid extraction technique has already been shown to recover the four 4'-DOX aglycones from serum with efficiency greater than 80% [16]. Over a concentration range of 20 ng/ml to 2 μg/ml (10—1000 ng injected on to the column) extraction efficiency of 4'-DOX was linear with r = 0.941 [actual concentration (y) = 1.3 × extracted concentration (x) − 4.7].

Analysis of patient serum

In Fig. 3B is a chromatogram of a serum extract of blood taken from a patient 20 min after receiving 30 mg/m^2 4'-DOX as an intravenous (i.v.) bolus injection. Peaks were identified which corresponded to 4'-DOX (173 ng/ml),

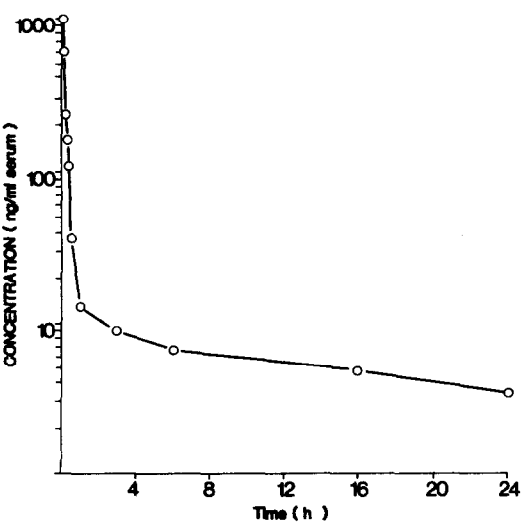


Fig. 4. Serum profile of 4'-deoxydoxorubicin in a patient after administration of 30 mg/m^2 as an i.v. bolus injection.

4′-DOL (6.7 ng/ml) and 4′-DOX-done (3.4 ng/ml). A chromatogram of a serum extract of blood taken from a patient 20 min after receiving 40 mg/m^2 ADR as an i.v. bolus injection is shown in Fig. 3C. Here, peaks corresponded to ADR (138 ng/ml), AOL (31 ng/ml), AOL-done (8 ng/ml) and ADR-done (49 ng/ml).

A patient serum profile of 4′-DOX is shown in Fig. 4. Pharmacokinetic parameters were calculated from an extended least-squares computer fit to the experimental data points contained in Fig. 4. The rate of disappearance of 4′-DOX from the serum fitted a bi-exponential decay indicative of a two-compartment open pharmacokinetic model where: serum concentration $c = 1120e^{-6.9t} + 8.4e^{-0.04t}$. Serum concentrations of 4′-DOX fell quickly immediately after its administration ($t_{1/2\alpha}$ 6 min) to a level of 12 ng/ml by 1 h. From 4 to 16 h it was necessary to extract from 2 ml serum and at 24 h 2 ml twice and combine extracts because the concentration of 4′-DOX in 1 ml of serum was less than the limit of detection of the HPLC assay (10 ng/ml). The $t_{1/2\beta}$ was calculated to be 18.8 h, $AUC_{0-\infty}$ (area under the curve) 387 ng/ml h and clearance 77.5 l/h/m^2. The serum profiles of 4′-DOL and 4′-DOX-done are to be found in Fig. 5. Both metabolites were present in serum at their peak concentrations after only 3 min and were no longer detectable by 30 min. The AUC of 4′-DOL was 12 ng/ml h and it accounted for 2.9% of the total concentration of serum 4′-DOX. The AUC of 4′-DOX-done was 2.3 ng/ml h and it accounted for only 0.6% of the total concentration of serum 4′-DOX.

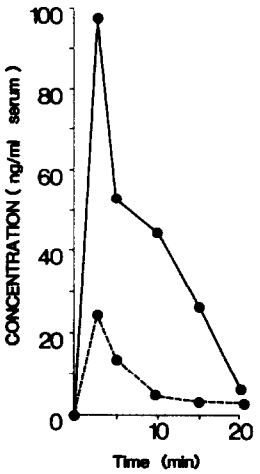


Fig. 5. Serum profiles of 4′-DOL (——) and 4′-DOX-done (— — —) in a patient after administration of 30 mg/m^2 4′-DOX as an i.v. bolus injection.

DISCUSSION

Preliminary reports of human metabolism of 4′-DOX suggest that it is converted to either 4′-DOL alone [17] or to 4′-DOL and the aglycone 4′-DOX-one [18]. The HPLC method described in this paper has been designed to clearly separate and identify the aglycone metabolites of 4′-DOX as well as the parent drug and 4′-DOL. In the patient studied the 7-deoxyaglycone of 4′-DOX (4′-DOX-done) was detected along with 4′-DOL. The behaviour of these two

metabolites was rather unusual: both were at their maximum serum concentration after only 3 min. To determine whether or not these metabolites could be chemical artefacts, purity and stability studies of 4′-DOX were carried out. These showed that 4′-DOX was 99.9% spectrophotometrically pure and that it did not degrade into aglycone or 4′-DOL artefacts but into non-fluorescent components. Presently, the metabolism of 4′-DOX is being investigated in our laboratory in a larger group of patients.

In vitro studies have shown that the 7-deoxyaglycone metabolites of ADR and AOL are by-products of semi-quinone free radicals [19, 20] whose generation by the heart has been implicated in causing ADR-induced cardiotoxicity [21, 22]. 7-Deoxyaglycone metabolites of ADR have been detected in cancer patients [15, 16] and their appearance has been cited as evidence that ADR free radical formation occurs in vivo in man [19]. As early as 1971 it was suggested that high aglycone metabolite levels were associated with ADR cardiotoxicity in animals [23, 24]. 5-Iminodaunorubicin, an analogue of ADR that does not cause appreciable cardiotoxicity in animals, is not metabolised to a 7-deoxyaglycone in vivo and is also not converted into free radicals in vitro [25]. A new, simple HPLC method which can detect 4′-DOX, 4′-DOL and low levels of the potentially important 7-deoxyaglycone metabolites will aid in the clinical evaluation of this useful new anthracycline.

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