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DETERMINATION OF ADRIAMYCIN, ADRIAMYCINOL AND THEIR 7-DEOXYAGLYCONES IN HUMAN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A reversed-phase isocratic high-performance liquid chromatographic assay is described for the measurement of adriamycin, adriamycinol and their 7-deoxyaglycones in human serum. The lower limit of detection in serum is 3 ng/ml for adriamycin and 1 ng/ml for adriamycinol and the 7-deoxyaglycones with coefficients of variation for k' of less than 5% throughout the day.

An extraction technique for serum is described which is capable of an almost equal recovery (> 77%) of adriamycin, metabolites and daunorubicin (the internal standard) without interference from endogenous components of serum. Serum concentrations of metabolites 15 min after intravenous bolus administration of 40 mg/m^2 adriamycin in two different patients were 26.5 and 16.6 ng/ml for adriamycinol; 109.8 and 5.8 ng/ml for the adriamycinol 7-deoxyaglycone and 21.4 and 17.1 ng/ml for the adriamycin 7-deoxyaglycone. A total of six metabolites of adriamycin were detected in the two patients using this methodology.

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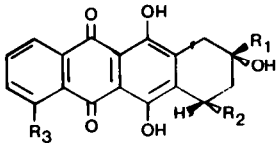
INTRODUCTION

Adriamycin (ADR) is a naturally occurring anthracycline glycosidic antibiotic with a broad spectrum of antitumour activity in human cancer [1]. In man, it was shown to be converted to at least eight different products, which were identified in urine using thin-layer chromatography (TLC) [2]. These were: adriamycinol (AOL), the major metabolite; five aglycones, adriamycin aglycone (ADR-ONE), adriamycin 7-deoxyaglycone (ADR-DONE), adriamycinol aglycone (AOL-ONE), adriamycinol 7-deoxyaglycone (AOL-DONE) and adriamycinol demethyl 7-deoxyaglycone (AOL-DM-DONE); and two conjugates of AOL-DM-DONE (Fig. 1). Pharmacokinetics of several aglycones were followed in fourteen cancer patients using TLC [3].

Recently, normal- and reversed-phase high-performance liquid chromatographic (HPLC) methods have been published for the separation of mixtures of ADR and metabolites [4—11]. Coupled to a variety of extraction techniques the only metabolite of ADR that was detected in patient serum or plasma by



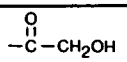
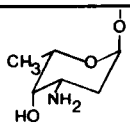
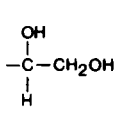
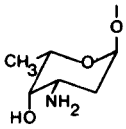
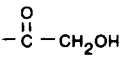
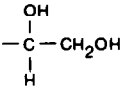
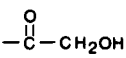
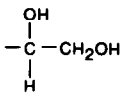
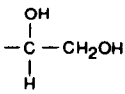
COMPOUND	R_1	R_2	R_3
ADR			
AOL			
ADR-ONE			
AOL-ONE			
ADR-DONE			
AOL-DONE			
AOL-DM-DONE			

Fig. 1. The structure of adriamycin and its metabolites.

HPLC was AOL [4, 7, 9, 12, 13]. The aglycones were suggested to be TLC artefacts caused by the hydrolysis of the glycosides ADR and AOL in situ during prolonged chromatographic development with acidic solvent systems [9, 12]. Whilst chemical decomposition may account for ADR-ONE and AOL-ONE, they can be produced by mild acid hydrolysis [10], it cannot explain the presence of 7-deoxyaglycone metabolites.

ADR and AOL 7-deoxyaglycone are formed by the chemical degradation of semi-quinone free radicals of ADR and AOL [14—16]. Since free radicals have been strongly implicated in the etiology of ADR-induced cardiotoxicity ADR-DONE and AOL-DONE may be important pharmacological markers. We report a new isocratic reversed-phase HPLC method with a simple extraction step which can detect adriamycin, adriamycinol and their 7-deoxy-aglycones in patient serum.

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 integrator (supplied by Scotlab Instrument Sales, Bellshill, U.K.) or a Rikadenki R-20 pen recorder (Rikadenki Mitsui Electronics, Chessington, U.K.) and μBondapak C_{18} prepacked columns 250 mm × 4.6 mm I.D. (supplied by HPLC Technology, Macclesfield, U.K.).

Reagents and standards

All methanol, acetonitrile, propan-2-ol and chloroform were HPLC reagent grade (Fisons Scientific, Loughborough, U.K.). Orthophosphoric acid and all other solvents and chemicals were of analytical reagent grade (AnalaR, BDH, Poole, U.K.). Water was double-distilled and deionised in a quartz glass still. Pure adriamycin—HCl and adriamycinol—HCl were a gift from Dr. S. Penco (Farmitalia, Milan, Italy). The internal standard, daunorubicin—HCl (DNR), was from May and Baker (Dagenham, U.K.). Adriamycin aglycone and adriamycin 7-deoxyaglycone were from Dr. Penco and were also synthesised. Daunorubicin aglycone (DNR-ONE), adriamycin aglycone and adriamycinol aglycone were all synthesised by mild acid hydrolysis with 0.1 *M* hydrochloric acid at 55°C for 1 h [10]. Adriamycin 7-deoxyaglycone and adriamycinol 7-deoxyaglycone were synthesised by catalytic hydrogenation using a palladium catalyst [2]. Purity of all the aglycones was assessed by TLC using 20 × 10 cm glass plates coated with a 250-μm layer of silica gel G (Analtech uniplates, Scotlab Instruments Sales) and three different ascending solvent systems (S). S 1 was chloroform—methanol—water (80:20:3); S 2 was chloroform—methanol—glacial acetic acid—water (80:20:14:6) and S 3 was ethyl acetate—ethanol—glacial acetic acid—water (80:10:5:5) [2]. The aglycones were visualised as orange spots under ultraviolet light at 254 nm. The chemical identity of methanolic solutions of all the synthesised aglycone

standards was verified by direct probe injection mass spectrometry with a Kratos MS 902S mass spectrometer and a DS 55C data system (Kratos Analytical Instruments, Manchester, U.K.).

Stock solutions (100 μg/ml) of AOL, ADR, DNR, AOL-ONE, ADR-ONE and DNR-ONE were prepared in methanol. Stock solutions of 10 μg/ml of ADR-DONE and AOL-DONE were prepared in methanol because of their poor solubility. All further dilutions were made with methanol to give a range of standard solutions of 0.025—10 μg/ml. All standard solutions were stored in PTFE-lined screw-capped bottles at −20°C and were made up fresh every month. Calibration curves were constructed by injecting 20 μl of standard solution to give a range of 0.5—200 ng on the column. All standard curves were linear ($r > 0.99$) and had intercepts at the origin. 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, 62.5% in methanol, acetonitrile and propan-2-ol, either 12.5:12.5:12.5 or 15:15:7.5, respectively, pH 3.2. Elution was isocratic at a flow-rate of 2.5 ml/min. Mobile phase was degassed by sonication at 12 μm for 15 min using a MSE sonicator (MSE Instruments, Crawley, U.K.). The column was at ambient room temperature (normally 25°C). Quantitation of sera extracts was by either peak area or peak height and was always by reference to standard calibration curves run on the same day.

Extraction procedure

Blood samples were collected from patients receiving 40 mg/m^2 ADR for the chemotherapy of cancer and were allowed to clot in plain glass tubes for 1 h at 4°C. Heparinised tubes were not used because heparin was shown to interfere with the HPLC assay. Blood was centrifuged at 1000 *g* for 10 min and the sera separated and stored at −20°C. Prior to extraction sera were thawed at room temperature.

To 1 or 2 ml of serum were added 100 ng DNR in 10 μl of methanol as an internal standard. The sera were then mixed with 5 vols. of chloroform—propan-2-ol (2:1) and either whirlimixed for two 1-min periods in 25-ml PTFE-lined screw capped test-tubes or vortexed for 30 min in 35-ml tapered glass centrifuge tubes (28 mm I.D.) with a Buchler vortex evaporator (Gallenkamp, East Kilbride, U.K.). After mixing, the samples were centrifuged at 2000 *g* for 15 min at 4°C to separate two phases. The upper aqueous phase was discarded by aspiration, the lower organic phase was transferred to a clean 35-ml tapered centrifuge tube and was evaporated to dryness in the Buchler vortex evaporator at 40°C and 25 mmHg of vacuum. The residue was redissolved in either 50 or 100 μl of methanol by whirlimixing for 1 min and 20 μl were injected onto the HPLC column.

RESULTS AND DISCUSSION

Physico—chemico properties of ADR and metabolites

An assumption which is commonly made regarding ADR and metabolites is

that they fluoresce all with the same quantum efficiency (Φ) and molar extinction coefficient (ϵ). To establish whether or not this is a valid assumption the fluorescence of each metabolite and DNR which was determined by HPLC was expressed relative to the fluorescence of equal concentrations of ADR (Table I). DNR and ADR were found to fluoresce equally but without exception the relative molar fluorescence (RMF) of the metabolites was greater than ADR. With AOL, ADR-ONE and AOL-ONE it was 1.5 times greater than ADR and with ADR-DONE and AOL-DONE it was 2 times greater than ADR (Table I). Assuming equal fluorescence is, therefore, likely to introduce large errors into quantitation when HPLC is involved [8, 11, 13, 17] and especially when only total fluorescent equivalents are measured [18—20].

TABLE I

PHYSICO—CHEMICO PROPERTIES OF ADRIAMYCIN AND METABOLITES

Compound	MW	Relative molar fluorescence*	Stability**		Percent solubility***		
			4°C	25°C	Water	Methanol	Chloroform
ADR	543.5	1	6.5	9.1	>10	>1	ns
AOL	545.6	1.5	3.6	6.3	>10	>1	ns
ADR-ONE	414.4	1.5	1.1	1.9	ns	>0.5	<0.02
AOL-ONE	416.4	1.5	<1	1.3	ns	>0.1	sparingly
ADR-DONE	398.4	2	1.6	1.3	ns	<0.002	sparingly
AOL-DONE	400.4	2	<1	<1	ns	<0.002	sparingly
DNR	527.5	1	6.1	8.0	>10	>1	ns

*Relative molar fluorescence is defined as the ratio of the integrated peak area of a metabolite and DNR over the integrated peak area of an equimolar concentration of ADR and is an expression of a metabolites fluorescence relative to the fluorescence of ADR.
**Stability data refer to the percentage of chemical decomposition per hour after a day at 25°C and per day after a week at 4°C of 10 μg/ml methanolic solutions.
***Solubility was determined using saturated or near saturated solutions after mixing for 4 h at room temperature; ns refers to not soluble and sparingly to solubility of less than 0.001%.

TLC has been implicated in producing ADR metabolite artefacts [9, 12]. Stability studies of ADR, metabolites and DNR were performed using the HPLC method described in this paper. At 4°C and 25°C the aglycones decomposed at less than 2% per day and per hour, respectively (Table I). ADR, DNR and AOL degraded faster than the aglycones at 3.6—6.5% per day at 4°C and at 6.3—9.1% per hour at 25°C. The glycosides (ADR, DNR and AOL) decomposed into non-detectable, non-fluorescent products and not into aglycones.

The final property studied was solubility. The aglycones and particularly the 7-deoxyaglycones were insoluble in water, methanol and chloroform (Table I). Further solvents were investigated to find one suitable. Acetonitrile, tetrahydrofuran, hexane, light petroleum (80—120°C), benzene and toluene were tried each with equally poor results. The alcohols methanol, ethanol and propan-2-ol gave the best results. A possible explanation for the poor solubility of the aglycones is that strong hydrogen bonding occurs between quinone and hydroxyl groups in the crystals lattice. Alcohols would compete for hydrogen

bonds which may account for their success as solvents. The glycosides were soluble in both water and to a lesser extent methanol but were insoluble in chloroform (Table I).

High-performance liquid chromatography

The separation achieved with the mixture of 12.5:12.5:12.5 methanol, acetonitrile and propan-2-ol is in Fig. 2A with capacity factors (k') and retention times (t_R) in Table II and with the mixture of 15:15:7.5 methanol, acetonitrile and propan-2-ol in Fig. 2B with k' and t_R in Table II. After equilibration with either mobile phase for 1 h k' varied by less than 5% over an 8-h period at 25°C. By reducing the proportion of propan-2-ol, keeping the phosphoric acid constant and increasing the methanol and acetonitrile equally k' can be increased without affecting the separation (compare Fig. 2A and B). Detection limits along with lowest recorded serum level in parenthesis are for AOL, 0.5 ng (2.1 ng/ml); AOL-ONE, 0.5 ng; ADR 1 ng (4.4 ng/ml); ADR-ONE,

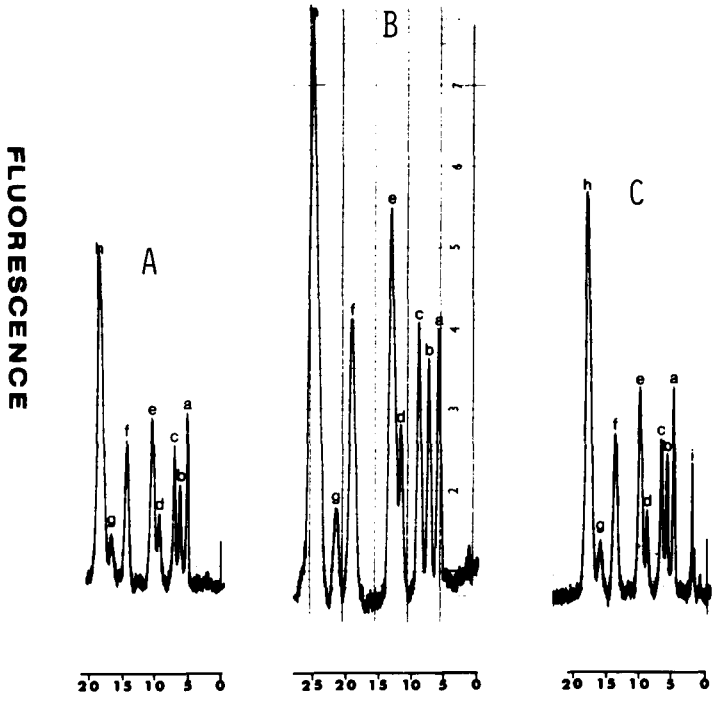


Fig. 2. Separation of a mixture of standards of ADR, metabolites and DNR (internal standard). (A) Chromatographic conditions: 5 m*M* (final concentration) orthophosphoric acid, 62.5% in methanol, acetonitrile and propan-2-ol, 12.5:12.5:12.5, pH 3.2. (B) Chromatographic conditions: 5 m*M* (final concentration) orthophosphoric acid, 62.5% in methanol, acetonitrile and propan-2-ol, 15:15:7.5, pH 3.2. (C) After extraction from human serum. Chromatographic conditions as in Fig. 2A. Elution rate 2.5 ml/min and fluorescence detection at 480 nm (excitation) and 560 nm (emission). Peaks: a = AOL; b = AOL-ONE; c = ADR; d = ADR-ONE; e = AOL-DONE; f = DNR; g = ADR—DONE, h = DNR-ONE; i = serum peaks.

TABLE II

SEPARATION OF ADRIAMYCIN AND METABOLITES BY ISOCRATIC REVERSED-PHASE HPLC USING μBONDAPAK C_{18}

Mobile phase 1 was 5 m*M* (final concentration) orthophosphoric acid, 62.5% in methanol, acetonitrile and propan-2-ol, 12.5:12.5:12.5. Mobile phase 2 was 5 m*M* (final concentration) orthophosphoric acid, 62.5% in methanol, acetonitrile and propan-2-ol, 15:15:7.5. The pH of both mobile phases was 3.2 and the elution rate was 2.5 ml/min. Fluorescence was measured at 480 nm (excitation) and 560 nm (emission).

Compound	Mobile phase 1		Mobile phase 2	
	k'	(t_R min)	k'	(t_R min)
AOL	2.1	(5.0)	2.4	(5.5)
AOL-ONE	2.6	(5.8)	3.3	(6.8)
ADR	3.6	(7.3)	4.1	(8.1)
ADR-ONE	4.2	(8.3)	6.0	(11.2)
AOL-DONE	5.3	(10.0)	6.7	(12.3)
DNR	7.8	(14.1)	10.1	(17.8)
ADR-DONE	8.7	(15.5)	12.8	(22.0)
DNR-ONE	9.4	(16.7)	13.6	(23.3)

1 ng (4.2 ng/ml); AOL-DONE, 0.5 ng (1.4 ng/ml); and ADR-DONE, 1 ng (2.7 ng/ml). Extraction of ADR and metabolites from serum did not introduce interference peaks (Figs. 2C and 3C).

In preliminary studies normal-phase HPLC was investigated for its ability to separate the aglycone metabolites of ADR and the glycoside metabolite AOL. Silica gel particles (5 and 10 μ*M*) (LiChrosorb Si 60, E. Merck, Darmstadt, F.R.G.) and a variety of chloroform-based mobile phases were tried. The aglycones always tended to elute early unresolved.

Negatively charged ions have been employed in the analysis of ADR and metabolites by HPLC [8, 21]. In one study [21] and in our own studies, these agents had little influence on k' of aglycone standards.

Separation of the aglycone metabolites of ADR has only been properly demonstrated using reversed-phase HPLC. In one case gradient elution was used [4], although this method was unable to resolve two of the aglycones, ADR-ONE and AOL-DONE. Another approach employed isocratic elution using one mobile phase to resolve the glycosides and another to resolve the aglycones and two internal standards [10]. A third method used isocratic elution with a single predominately aqueous mobile phase with the consequence that retention times of the later eluting peaks were delayed and poor peak symmetry and decreased sensitivity resulted [7].

The present method achieves a separation of ADR and its major glycoside metabolite AOL as well as four different aglycone metabolites and DNR without requiring gradient elution or more than one mobile phase. Also, a complete analysis can be performed in under 18 min without broadening of later eluting peaks and analysis time can be controlled by changing the proportions of the different components of the mobile phase.

Serum extractions

Over 50 control extractions with blood bank serum were performed. 1 or 2 ml of serum were spiked with 10 μl of a methanolic solution containing 10—1000 ng of standard. Individual extraction efficiencies ± S.D. expressed as a percentage for 10 ng of standard were for AOL, 78.4 ± 12.3%; AOL-ONE, 84.8 ± 9.3%; ADR, 83.4 ± 6.1%; ADR—ONE, 85.8 ± 8.6%; AOL-DONE, 91.0 ± 5.3%; DNR, 77.8 ± 7.4%; and ADR-DONE, 82.1 ± 9.6%. This simple technique did not involve prolonged treatment with ethanolic 0.6 *M* hydrochloric acid [7] or require high-speed centrifugation to separate phases after precipitating proteins with ammonium sulphate [4] or use prepacked mini-columns [12] and was capable of more or less equal recovery of ADR and metabolites along with DNR. A typical blank serum extract is shown in Fig. 3C.

Fig. 3A and B shows chromatograms of blood taken from two different cancer patients 15 min after intravenous bolus administration of 40 mg/m^2 ADR. In the first patient (Fig. 3A) four ADR metabolite peaks were identified

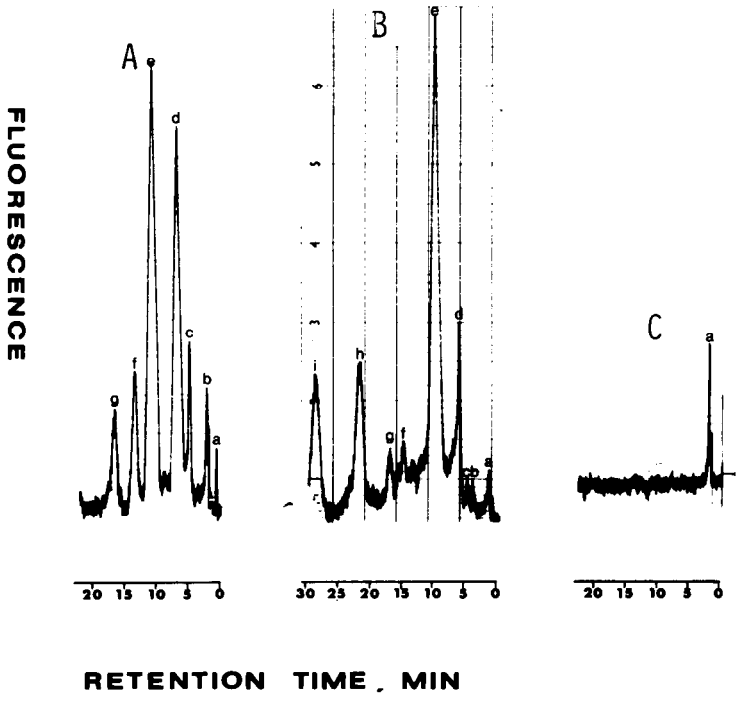


Fig. 3. (A) Serum extract from a patient who received ADR as an intravenous bolus of 40 mg/m^2. Chromatographic conditions as in Fig. 2A. Peaks: a = serum peaks; b = ADR metabolite (k' 1.8); c = AOL (26.5 ng/ml); d = ADR (257.6 ng/ml); e = AOL-DONE (109.8 ng/ml); f = DNR (internal standard); and g = ADR-DONE (21.4 ng/ml). (B) Serum extract from a second patient who received ADR as an intravenous bolus of 40 mg/m^2. Chromatographic conditions: 5 m*M* (final concentration) orthophosphoric acid, 62.5% in methanol, acetonitrile and propan-2-ol, 16.5:16.5:4.5, pH 3.2. Elution rate 2.5 ml/min and detection as in Fig. 2A. Peaks: a = serum peaks; b = ADR metabolite (k' 1.5); c = ADR metabolite (k' 1.8); d = AOL (16.6 ng/ml); e = ADR (260 ng/ml); f = ADR-ONE (4.1 ng/ml); g = AOL-DONE (5.8 ng/ml); h = DNR (internal standard) and i = ADR-DONE (17.1 ng/ml). (C) Blank serum extract. Chromatographic conditions as in Fig. 2A. Peaks: a = serum peaks.

apart from the parent drug and the internal standard. The metabolites present were AOL (26.5 ng/ml), AOL-DONE (109.8 ng/ml), ADR-DONE (21.4 ng/ml) and an early eluting metabolite with k' of 1.8. In the second patient (Fig. 3B) six ADR metabolite peaks were identified apart from the parent drug and the internal standard. The metabolites present were AOL (16.6 ng/ml), AOL-DONE (5.8 ng/ml), ADR-ONE (4.1 ng/ml) and ADR-DONE (17.1 ng/ml) along with two early eluting metabolites with k' of 1.5 and 1.8.

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