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Note

Estimation of doxorubicin and doxorubicinol by high-performance liquid chromatography and advanced automated sample processor

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Doxorubicin (adriamycin) is an anthracycline antibiotic with a wide range of antitumour activity [1], although its use is limited by acute myelotoxicity and the risk of irreversible cardiomyopathy. Its toxicity is particularly troublesome in patients with hepatic impairment, since appropriate rules for dose modification have not been established [2]. One approach could be to use a low-dose test infusion to predict drug disposition prior to administration of a therapeutic dose [3]. Such a strategy requires a rapid, accurate and sensitive technique for estimation of doxorubicin and its active metabolite, doxorubicinol.

Doxorubicin and many of its metabolites are naturally fluorescent and so have been estimated in plasma by high-performance liquid chromatography (HPLC) followed by fluorescence detection [4–8]. We have, until recently, measured doxorubicin and doxorubicinol by a technique based on one of these methods [6] involving extraction of plasma with a chloroform–propan-2-ol mixture, followed by chromatography on a bonded-silica phenyl column. Although this method works reasonably well, the sample preparation is time-consuming, and the realistically achievable detection limit in routine use of 4–5 ng/ml was insufficient to estimate doxorubicin in plasma samples taken late (up to 72 h after dosing) in pharmacokinetic studies. The acquisition of an AASP® (advanced automated sample processor, Varian Assoc.) suggested a different approach to sample preparation that could improve both the speed and sensitivity of the assay.

The AASP is an instrument that introduces a cartridge containing about 50 mg of a bonded-silica sorbent into the solvent stream of a high-performance liquid chromatograph. With the appropriate conditions, nearly all of the drug extracted from plasma by such a column is injected into the chromatograph. This

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improves sensitivity as compared with a classical solvent extraction where often only one third to one fifth of the final extract is actually injected.

EXPERIMENTAL

Chemicals

Doxorubicin, doxorubicinol and daunorubicin were gifts from Farmitalia (Milan, Italy). All other chemicals were obtained from BDH (Poole, U.K.) and were of AnalaR grade, except for the acetonitrile and water, which were of HPLC grade.

Instrumentation

The HPLC system consisted of a Shimadzu RF530 fluorescence detector, a Shimadzu LC3A pump and Shimadzu CR1B computing integrator (Dyson Instruments, Hetton, U.K.). The column used was a 10-μm μBondapak Phenyl, 30 cm$\times$3.9 mm (Millipore, Harrow, U.K.).

The C_2 ethyl cassettes for the AASP were obtained from Jones Chromatography (Hengoed, U.K.) and the AASP from Varian Assoc. (Walton-on-Thames, U.K.). A second fluorescence detector was also used in some of our studies: the Merck-Hitachi F1000 (Biard and Tatlock, Dagenham, U.K.).

Assay conditions

The mobile phase was acetonitrile–0.1 *M* ammonium formate buffer, pH 4.0 (31:69). The flow-rate was 1.5 ml/min. The excitation wavelength was 470 nm and the emission wavelength was 565 nm. The AASP run and cycle times were set at 12 min with the valve reset at 1 min after injection.

Sample preparation

The C_2 cassettes were prepared on the modified Vac-Elut station as supplied with the AASP. Various solutions were perfused through the cassettes with positive pressure (0.34 bar) from a nitrogen cylinder. The cartridges were conditioned by passing through, in order, 1 ml of acetonitrile, 0.5 ml of water and 0.5 ml of 0.1 *M* ammonium formate buffer, pH 4.0. Then 0.5 ml plasma mixed with 0.5 ml water was passed through the cartridges followed by a 0.5-ml water wash. These cartridges were introduced into the HPLC system via the AASP. Before extraction all plasma was centrifuged at 10 000 *g* for 3 min (Eppendorf Microfuge, Anderman, Kingston-upon-Thames, U.K.) to prevent blocking of the cartridge by particulate matter.

Preparation of calibration curves

Calibration curves were prepared by spiking blank plasma with 2000–2 ng/ml doxorubicin and 300–2 ng/ml doxorubicinol. The internal standard, daunorubicin (200 ng/ml) was added to all plasma samples and standards. Peak heights were measured by the integrator and used to calculate peak-height ratios for the drug and metabolite.

TABLE I

INTRA-ASSAY AND INTER-ASSAY REPRODUCIBILITY AND RECOVERY OF DOXORUBICIN AND DOXORUBICINOL FROM PLASMA

Compound	Concentration added (ng/ml)	*n*	Concentration measured (mean ± S.D.) (ng/ml)	Coefficient of variation (%)	Recovery (mean ± S.D.) (%)
Intra-assay					
Doxorubicin	2	7	2.0 ± 0.1	7.3	91.8 ± 3.6
	10	9	10.0 ± 0.3	2.7	73.5 ± 6.6
	100	7	97.5 ± 1.7	1.8	65.6 ± 1.6
	500	8	502.2 ± 31.8	6.3	71.7 ± 3.4
	1000	8	998.9 ± 32.7	3.3	80.1 ± 1.3
Doxorubicinol	2	7	2.0 ± 0.1	6.4	82.8 ± 3.7
	10	9	10.1 ± 0.4	4.1	78.9 ± 4.4
	20	7	18.7 ± 2.7	1.4	59.0 ± 2.1
	100	8	111.3 ± 9.1	8.2	79.4 ± 2.8
	200	8	194.8 ± 7.1	3.6	81.5 ± 1.7
Inter-assay					
Doxorubicin	10	7	10.2 ± 0.25	2.5	
	500	5	472.0 ± 29.6	6.3	
	1000	5	1097.0 ± 67.1	6.1	
Doxorubicinol	10	6	10.2 ± 0.4	3.9	
	200	8	210.0 ± 13.7	6.4	
	300	5	291.0 ± 8.7	3.0	

RESULTS AND DISCUSSION

Linear calibration curves were obtained up to 2000 ng/ml for doxorubicin and 300 ng/ml for doxorubicinol. The inter- and intra-assay coefficients of variation and the recovery from plasma were satisfactory for both compounds, and are shown in Table I.

Previous assays have claimed detection limits for doxorubicin of between 2 and 4 ng/ml as based on signal-to-noise ratios of 1:2 or 1:3 [4,5,7]. Use of a solid-phase sample preparation followed by automated injection of the extraction cassettes has significantly improved on this. Concentrations of 2 ng/ml doxorubicin and 2 ng/ml doxorubicinol can now be accurately and routinely measured with acceptable intra-assay coefficients of variation of 7.3 and 6.4%, respectively. This improved sensitivity is demonstrated in Fig. 1C by a chromatogram obtained from a patient sample taken 72 h after a dose of doxorubicin (50 mg/m^2) which would not have been measureable by our previous method.

Fig. 2 shows an example of the plasma concentration profile obtained from a patients receiving single-agent intravenous doxorubicin therapy.

Sensitivity could be improved further by using a different fluorescence detector (Merck-Hitachi F1000) that utilises a more powerful (150 W) xenon lamp, and by extracting 1 ml of plasma. Fig. 1D shows a chromatogram produced under

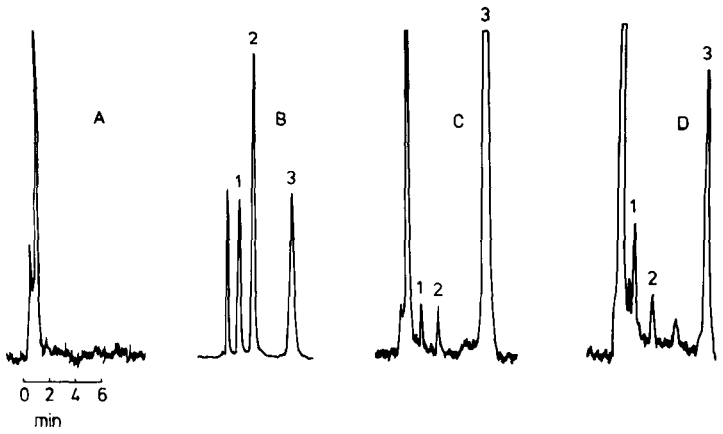

Fig. 1. Representative chromatograms obtained from plasma samples for doxorubicin and doxorubicinol. (A) Blank plasma from patient prior to treatment with doxorubicin. (B) Blank plasma spiked with 50 ng/ml doxorubicin, 100 ng/ml doxorubicinol and internal standard. (C) Patient sample 72 h after doxorubicin dose (50 mg/m^2) containing 1.5 ng/ml doxorubicin and 3.1 ng/ml doxorubicinol. (D) Extraction of 1 ml of plasma containing 400 pg/ml doxorubicin and 400 pg/ml doxorubicinol, using the Merck-Hitachi F1000 fluorescence detector. Peaks: 1 = doxorubicinol; 2 = doxorubicin; 3 = daunorubicin, internal standard.

these conditions from a plasma sample spiked with 400 pg/ml each of doxorubicin and doxorubicinol.

This method also significantly improves sample throughput allowing at least thirty samples and standards to be processed in a working day. In particular, the

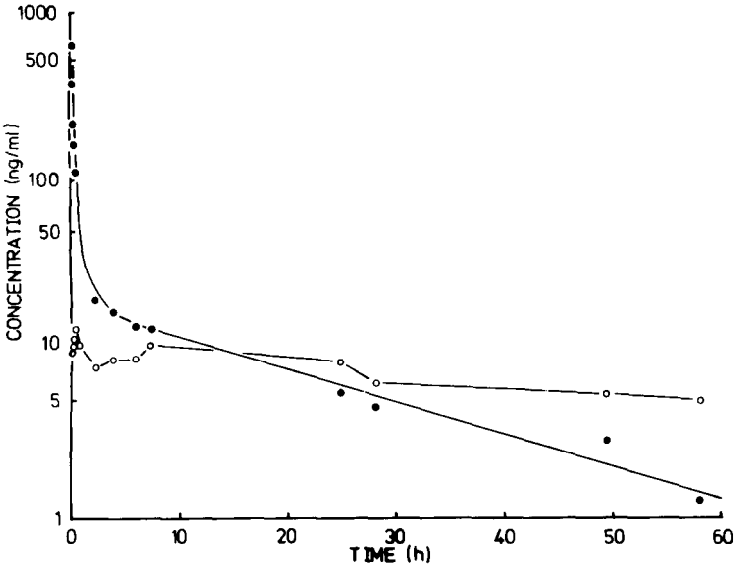

Fig. 2. Plasma concentration-time profiles of doxorubicin (●) and doxorubicinol (○) following intravenous administration of doxorubicin (30 mg/m^2) to a patient. The terminal half-life of doxorubicin was 15.3 h.

time-consuming centrifugation, solvent transfer and drying-down procedures of other methods [2,4–6] are avoided. This technique should also be easily extended to assay of anthracyclines in tissue extracts. The solid phase extraction procedure developed could, in addition, be modified for use with manual injection into a high-performance liquid chromatograph.

The only disadvantage of the assay appears to be the cost of the instrumentation and disposables involved.

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