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Measurement of epidoxorubicin and its metabolites by high-performance liquid chromatography using an advanced automated sample processor

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ABSTRACT

A sensitive and rapid method for measuring epidoxorubicin and its six metabolites by high-performance liquid chromatography using an advanced automated sample processor is described. Plasma samples (1 ml) were extracted using C_2 cassettes, and reversed-phase chromatography was performed with an Apex II ODS column. The isocratic mobile phase of acetonitrile–0.019 M NaH_2PO_4 (pH 4.0) had a flow-rate of 1 ml/min and the fluorescence detector an excitation wavelength of 480 nm with an emission at 580 nm. Linear calibration curves were obtained which were reproducible both within-day and day-to-day (coefficients of variation < 10%). The extraction efficacy of epidoxorubicin was 88% and ranged from 51 to 88% for the metabolites. This method has been successfully applied to measure the plasma levels of these compounds in patients receiving epidoxorubicin over a wide dose range (12–120 mg/m^2) and in patients with disturbed liver biochemistry.

INTRODUCTION

The anthracycline epidoxorubicin (EPI) is a derivative of doxorubicin (DOX) recently introduced into clinical practice. EPI differs from DOX only in the orientation of the C-4 hydroxyl group of amino sugar moiety [1], but this difference significantly influences the metabolism of EPI [2]. Clinical trials have indicated that EPI may have a higher therapeutic index and be associated with less cardiotoxicity than DOX [3–5].

Earlier methods for measuring EPI and its metabolites by high-performance liquid chromatography (HPLC) from biological fluids have used solvent extraction [6,7] which is time-consuming and can give both variable and relatively low recoveries. More recent analytical methods have used a preparative liquid–solid extraction step [8–10] which has improved the recovery but still involves relatively lengthy sample preparation. The incorporation of an advanced automated sample processor (AASP) into the HPLC system obviates these potential problems improving both the sensitivity and speed of the assay. The drug and metabolites are extracted onto a cartridge containing bonded silica sorbent which is intro-

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duced into the solvent stream of an HPLC system. Under appropriate conditions approximately 98% of the drug extracted from plasma is automatically injected into the chromatograph.

Using this method to measure EPI and metabolites, the sample throughput has been significantly increased allowing up to twenty samples and standards to be processed in a working day. Moreover, the compounds can be measured routinely and reproducibly down to levels of 1 ng/ml from only 1 ml of plasma on a single isocratic run of only 22 min.

EXPERIMENTAL

Chemicals

EPI, epidoxorubicinol (Eol), epidoxorubicin glucuronide (E-glu), epidoxorubicinol glucuronide (Eol-glu), 7-deoxyadriamycinone (aglycone A), 13-dihydroadriamycinone (aglycone B), 7-deoxy-13-dihydroadriamycinone (aglycone C) and daunorubicin (DRN, used as the internal standard) were kindly provided by Farmitalia Carlo Erba (Milan, Italy). All of the reagents were of AnalaR grade, except for acetonitrile which was of HPLC grade (BDH, Poole, UK). Purified water was obtained from a Milli-Q water system (Millipore, Molsheim, France).

Instrumentation

Chromatographic analysis was performed on a HPLC system consisting of a Shimadzu LC3A pump and a Shimadzu CR1B computing integrator (Dyson Instruments, Hetton, UK) with a Merck-Hitachi F1000 fluorescence detector (Baird and Tatlock, Dagenham, UK). The pre-column used was a LiChrosorb RP-18, 10 μm (5 cm × 5 mm) and the analytical column was Apex II ODS, 5 μm (10 cm × 5 mm) (Jones Chromatography, Hengoed, UK).

The AASP and cassette preparation station were obtained from Varian Assoc. (Walton-on-Thames, UK) and the C_2 cassettes were from Jones Chromatography.

Assay conditions

The mobile phase comprised 0.019 M NaH_2PO_4 (adjusted to pH 4.0 with 0.1 M orthophosphoric acid)–acetonitrile (2.25:1, v/v) with a flow-rate of 1 ml/min. The purge solvent (buffer A) was 0.019 M Na_2PO_4 (pH 4.0)–acetonitrile (9:1) and had a pre-purge volume of 75 μl and a post-purge volume of 500 μl. The AASP run and cycle times were set at 22 and 25 min, respectively, and the valve was reset at 1 min following injection. The excitation wavelength for fluorescence detection was 480 nm and the emission wavelength was 580 nm.

Sample preparation

Stock solutions of EPI, Eol, E-glu and Eol-glu were prepared in purified water and stored at 4°C. The aglycone metabolites were poorly soluble in water, so they

were reconstituted in dimethylsulphoxide (DMSO) and stored at 20°C. All solutions were protected from light. Further dilution to provide a range of concentrations was achieved using purified water.

The C_2 cartridges, of which each cassette has ten, were prepared on a AASP cassette preparation station. Before addition of the plasma sample, the cassette was consecutively rinsed with 1 ml of methanol, 0.5 ml of water and 0.5 ml of buffer A under positive pressure (0.34 bar) from an air cylinder. Next, 1 ml of spiked or patient plasma was mixed with 0.5 ml of water and passed through the cassette followed by 0.5 ml of buffer A. The prepared cassette was then introduced into the AASP and thereby into the solvent stream of the HPLC system.

Preparation of calibration curves

Calibration curves were prepared by adding known amounts of EPI and metabolites to normal plasma to give a range of concentrations for EPI of 1–2000 ng/ml, for E-glu of 1–500 ng/ml and for the remaining compounds of 1–250 ng/ml. The internal standard, DRN (100 ng/ml), was added to all plasma samples and standards. Peak heights were measured by the integrator and used to calculate the peak-height ratios for the drug and metabolites.

RESULTS AND DISCUSSION

Injection of the isolated EPI and six metabolites from the C_2 cassettes into the chromatographic system allows efficient and complete resolution of the compounds in a single isocratic run of 22 min. No interfering peaks were seen when processing blank plasma. Fig. 1 shows a typical chromatogram obtained from 1

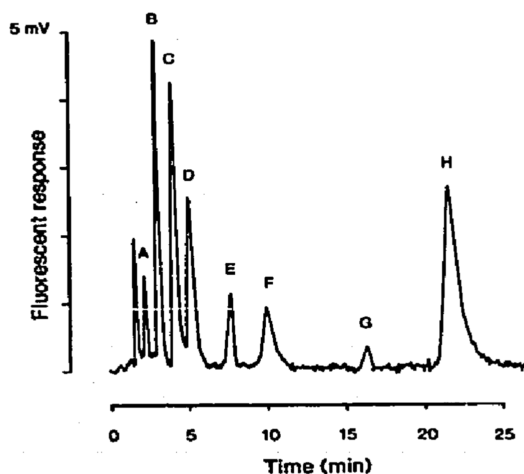

Fig. 1. Chromatogram from a 1-ml plasma sample spiked with 5 ng of epidoxorubicinol glucuronide (A), epidoxorubicin glucuronide (B), 13-dihydroadriamycinone (C), epidoxorubicinol (D), 7-deoxy-13-dihydroadriamycinone (E), epidoxorubicin (F), 7-deoxyadriamycinone (G) and the internal standard, daunorubicin (H).

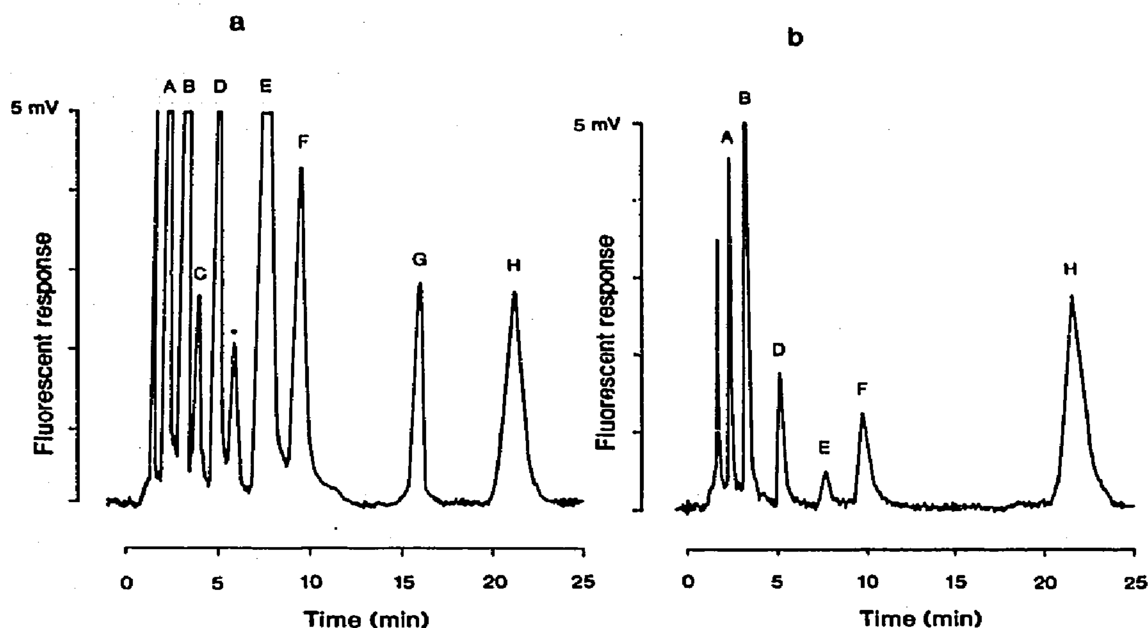

Fig. 2. (a) Patient sample 2 h after an EPI dose of 12.5 mg/m². Peaks defined as for Fig. 1 (the asterisk indicates an additional peak in the position of adriamycinone due to chemical degradation). (b) Patient sample 48 h after an EPI dose of 12.5 mg/m². Peaks defined as for Fig. 1.

ml of normal plasma spiked with EPI and all six metabolites (5 ng/ml). Examples of chromatograms obtained at 2 and 48 h after injection from a patient receiving 12.5 mg/m² EPI are given in Fig. 2a and b, respectively. Plasma concentration–time profiles for EPI and metabolites from a patient receiving 75 mg/m² EPI are given in Fig. 3.

The extraction efficacy of the compounds from plasma was calculated by comparing the peak-height ratios in the chromatograms of extracted samples with

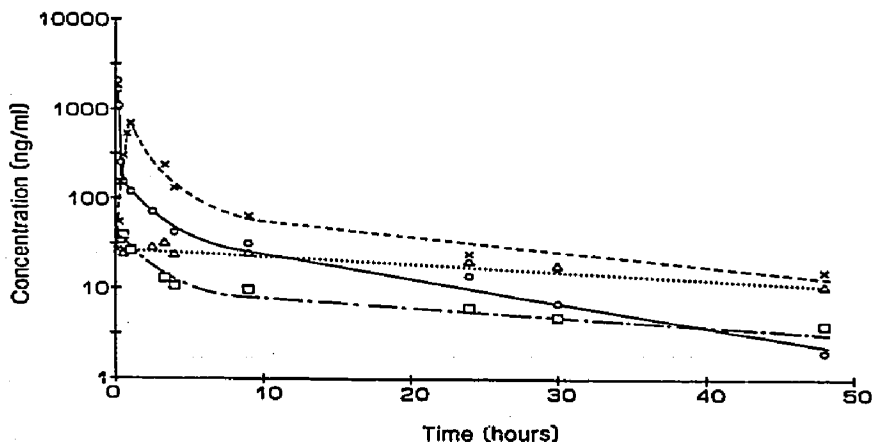

Fig. 3. Plasma profiles of (○) epidoxorubicin, (×) epidoxorubicin glucuronide, (△) epidoxorubicinol and (□) 7-deoxy-13-dihydroadriamycinone from a patient after treatment with 75 mg/m² epidoxorubicin.

TABLE I

RECOVERY OF EPIDOXORUBICIN AND ITS METABOLITES FROM PLASMA

Compound	Concentration range (ng/ml)	Mean recovery (%)
EPI	5–2000	83
Eol	5–500	62
E-glu	7–350	79
Eol-glu	3–150	83
Aglycone A	5–100	51
Aglycone B	5–100	88
Aglycone C	5–75	57

those of standards of identical concentrations prepared in mobile phase (Table I). The mean recovery of EPI was 83%, and ranged from 51 to 88% for the six metabolites.

Linear calibration curves ($r^2 > 0.995$), calculated b[illegible]e least-squares method,

TABLE II

WITHIN-DAY PRECISION STUDY

Compound	Concentration added (ng/ml)	Mean concentration measured (n = 6) (ng/ml)	C.V. (%)
EPI	2000.0	2002.0	4.7
	100.0	98.6	3.9
	50.0	50.0	4.8
	5.0	5.8	3.1
Eol	100.0	100.0	3.1
	50.0	50.0	7.6
	10.0	8.8	2.3
E-Glu	350.0	351.0	6.4
	70.0	78.4	2.5
	35.0	36.7	4.2
	3.5	3.5	5.7
Eol-glu	150.0	150.0	6.2
	30.0	34.3	2.1
	15.0	15.2	3.9
	3.0	3.8	2.6
Aglycone A	500.0	521.0	5.1
	75.0	77.3	3.2
Aglycone B	100.0	101.5	2.7
	10.0	9.3	2.5
Aglycone C	500.0	534.0	5.0
	75.0	79.3	7.2

TABLE III

DAY-TO-DAY PRECISION STUDY

Compound	Concentration added (ng/ml)	Mean concentration measured (n = 6) (ng/ml)	C.V. (%)
EPI	2000.0	2035.0	3.5
	100.0	99.0	4.6
	50.0	50.1	2.2
	5.0	5.6	5.2
Eol	100.0	102.9	7.6
	50.0	49.9	3.6
	10.0	9.8	5.7
E-glu	350.0	359.0	5.7
	70.0	77.8	4.3
	35.0	36.7	4.2
	3.5	3.8	5.0
Eol-glu	150.0	147.0	6.6
	30.0	31.9	3.2
	15.0	14.3	4.6
	3.0	2.9	7.9
Aglycone A	500.0	505.4	4.4
	75.0	74.4	3.9
Aglycone B	100.0	101.2	2.0
	10.0	9.7	6.3
Aglycone C	500.0	504.8	6.1
	75.0	71.5	7.6

were obtained up to 2000 ng/ml for EPI, to 350 ng/ml for E-glu, to 150 ng/ml for Eol-glu and to 500 ng/ml for the remaining metabolites. The within-day precision of the assay was studied by repeating the analysis of six plasma samples spiked with EPI and metabolites at concentrations ranging from 1.5 to 2000 ng/ml six times in one day (Table II). The day-to-day precision of the assay was calculated by comparison of six standard curves, prepared over the same concentrations as for the within-day study and analysed on consecutive days (Table III). For both the within-day and day-to-day precision studies, coefficients of variation (C.V.) were less than 10% for all compounds.

The routine detection limit of the assay, defined as a signal-to-noise ratio of 3:1, ranged from 0.5 ng/ml for the glucuronides to 1 ng/ml for EPI and the aglycones when using 1 ml of plasma. These levels of detection were adequate for the routine measurement of EPI and its metabolites in the plasma of patients 72 h after receiving doses of between 12.5 and 120 mg/m^2. Greater sensitivity can be achieved using a larger volume of plasma.

In conclusion, the incorporation of an AASP (sorbent extraction) into the HPLC system has resulted in a sensitive and rapid method for the identification

and quantitation of EPI and its metabolites from 1 ml of plasma. These metabolites include the poorly water-soluble aglycones A and C which we dissolved in DMSO and whose dissolution has not been detailed in previous methods. Accurate measurement of low plasma levels of these compounds has allowed pharmacokinetic studies to be carried out under a wide range of circumstances. These include patients treated with low-dose chemotherapy given as a bolus injection or those receiving treatment by infusion where a low detection limit is required. In addition, the analytical method is applicable to the measurement of plasma levels up to 72 h after treatment in patients receiving 12.5–120 mg/m^2 EPI by bolus injection and also in those with disturbed liver biochemistry [11]. The rapidity of the method allows both the extraction and chromatographic analysis of up to twenty plasma samples a day, an important consideration if pharmacokinetic data are to be used in the monitoring or planning of treatment regimens.

The method could, with minor modification, be applied to the measurement of EPI and metabolite levels in urine, bile, tissue and cell extracts to provide a better understanding of its metabolism and distribution.

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