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Determination of plasma concentrations of epirubicin and its metabolites by high-performance liquid chromatography during a 96-h infusion in cancer chemotherapy

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Abstract

In order to determine epirubicin and its metabolites at low concentrations (<38 ng/ml) in small plasma samples, a fast reliable method based on a precipitation pre-treatment and sensitive reversed-phase isocratic HPLC has been developed and validated for epirubicin in the range 5–100 ng/ml. The R.S.D. was 5–9% over this concentration range. For human serum containing 25 ng/ml of epirubicin, the inter- and intra-day variation was <10%. Recoveries of the metabolites epirubicinol, 7-deoxydoxorubicinone and 7-deoxydoxorubicinolone at 20 ng/ml ranged from 94–104%. The assay has been used to study human plasma samples taken during a 96-h infusion of epirubicin in a patient with multiple myeloma. The combined levels of the unseparated metabolites, epirubicin glucuronide and epirubicinol glucuronide, were semiquantitatively determined after treatment with β-glucuronidase. The metabolites epirubicinol and 7-deoxydoxorubicinolone, but not 7-deoxydoxorubicinone, were also detected and measured.

Keywords: Epirubicin; Epirubicinol; 7-Deoxydoxorubicinone; 7-Deoxydoxorubicinolone; 4′-Epidoxorubicinone; Doxorubicinone; Doxorubicinolone

1. Introduction

Epirubicin (4′-epidoxorubicin) is a relatively new anthracycline antibiotic and antitumour derivative of doxorubicin that has been used to treat a wide range of cancers. Epirubicin differs from doxorubicin in the inversion of stereochemistry at C-4′ (daunosamine ring). Although the orientation of the hydroxyl group at this position is equatorial in epirubicin and axial in doxorubicin, this deceptively small change does not apparently change the therapeutic efficacy of the drug. Epirubicin is much less cardiotoxic than doxorubicin [1]. These drugs are extensively metabolised in vivo. Unlike doxorubicin, epirubicin is conjugated with glucuronic acid [2,3]. The pharmacology of doxorubicin is independent of the method of administration [4,5].

Continuous infusion of doxorubicin has been proposed as a potentially advantageous means of administration because the absence of a high peak

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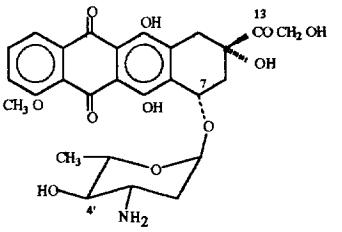
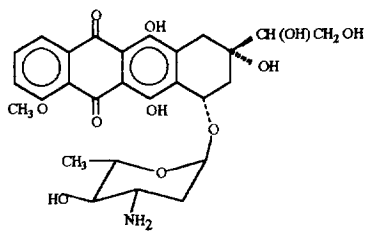
concentration of the drug may minimise cardiotoxicity whilst maintaining drug exposure [6]. The observation that adriamycin (doxorubicin) has reduced toxicity against a cell line in culture as a multicellular spheroid compared to the same line as a monolayer [7] also raises the possibility that prolonged exposure may be of value in treating tumour deposits, where variations in drug penetration may be relevant.

A potential benefit of infused anthracycline chemotherapy has been identified in breast cancer [8], lymphomas [9] and in multiple myeloma [10]. These studies have been based on doxorubicin; however, little is known about the effect of varying the administration schedule of epirubicin. The primary objective of the present work was to develop methods to study this.

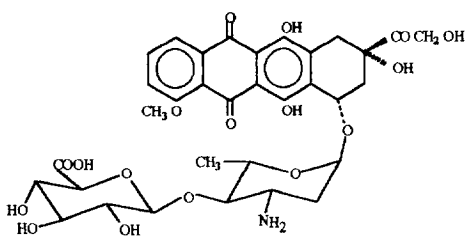
The requirements of this study were to find an analytical procedure that is rapid, robust and has a high sensitivity for epirubicin and its metabolites (Fig. 1) in clinical plasma samples. The polarity of anthracyclines varies considerably. For example, the glucuronides are considerably more polar than 7-deoxydoxorubicinone, the least polar of the major metabolites of epirubicin and doxorubicin. HPLC is the analytical technique of choice for these molecules and several methods have been published [11–16]. The analytical method chosen for this study is modified from that developed by Nicholls et al. [11]. Our optimised method provides the desired speed



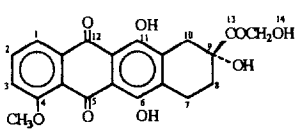
Epirubicin



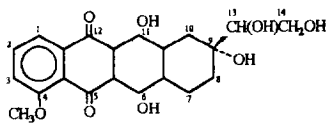
Epirubicinol (13-S-dihydroepirubicin)



Epirubicin glucuronide
(4'-O-β-D-glucuronyl epirubicin)



7-Deoxydoxorubicinone



7-Deoxydoxorubicinolone

Fig. 1. Structures of EPI and its major metabolites.

and a high degree of separation for a wide range of epirubicin and doxorubicin derivatives.

The pre-treatment method applied to horse serum by Nicholls et al. [11] was found to yield very low recoveries of epirubicin and its metabolites at low concentrations of clinical relevance i.e. <50 ng/ml. This method also required an impractically large sample size (minimum of 1 ml). An alternative sample pre-treatment method was therefore examined, based on an earlier method developed by Brown et al. [12], in order to meet the exacting requirements of the present study. This simple precipitation method permits small plasma samples (0.1–0.2 ml) to be analysed, with concentrations of epirubicin as low as 10 ng/ml (17 pmol/ml).

The objective of the present work was to establish a validated analytical protocol for the assay of epirubicin and its principal metabolites in order to obtain preliminary pharmacokinetic and metabolism data on a 96-h infusion schedule in a mixed group of cancer patients.

2. Experimental

2.1. Reagents and materials

Methanol (CH_3OH) and acetonitrile (CH_3CN) were of HPLC grade and were purchased from Fisons Scientific Equipment (Loughborough, UK). Phosphate buffer was prepared from disodium hydrogenphosphate (60 m*M*) (Fisons Scientific Equipment) and triethylamine (TEA, 0.05%, v/v) (Merck, Poole, UK), adjusted to pH 4.2 with citric acid (Merck). The mobile phase consisted of CH_3CN–60 m*M* phosphate buffer containing 0.05% (v/v) TEA, pH 4.2 (35:65, v/v). The precipitation solvent used in the plasma pre-treatment was composed of acetonitrile (80%) and 0.1 *M* orthophosphoric acid (20%) (Merck).

Epirubicin (EPI) (Pharmorubicin) and metabolites were kindly provided by Pharmacia (Milan, Italy); these were epirubicinol (EOL), doxorubicinone, doxorubicinolone, 7-deoxydoxorubicinone (DD) and 7-deoxydoxorubicinolone (DDL). Pooled human male AB (whole blood) serum (Cat No: H1513) and β-glucuronidase enzyme were purchased from Sigma (Poole, UK). Heparinised saline (Hepflush) was used, as received from Leo Laboratories (Aylesbury, UK).

2.2. Sample collection protocol

With Ethical Committee approval, blood samples (2–3 ml) were taken into lithium heparin on five consecutive days from a cancer patient with multiple myeloma, at Airedale General Hospital (West Yorkshire, UK). A sample was taken before treatment on day 0. Four samples were then taken on each of the next four consecutive days during a continuous infusion of EPI. The drug in water was infused into a main chest vein by an ambulatory infusion pump (CADD-1, Pharmacia Deltec, Milton Keynes, UK) at the rate of 22.5 mg/m^2 per day. The total dose given over four days was 162 mg (90 mg/m^2). Blood samples were centrifuged immediately and the plasma stored at −20°C in the dark.

2.3. HPLC system

A modular HPLC system was used comprising of a Gilson 305 10SC pump (Villiers-le-Bel, France) which delivered mobile phase at a flow-rate of 1 ml/min through an Spherisorb ODS1 column (250× 4.6 mm I.D.) packed with C_{18} Spherisorb (5 μm) supplied by Hichrom (Reading, UK). A "direct-connect" guard column (1 cm) (Alltech, Carnforth, UK) with C_{18} packing was fitted to the inlet of the column. The detector was a Gilson 121 fluorometer provided with a 480-nm excitation filter and an emission filter at 560 nm. A Rheodyne injector provided with a 20-μl loop was used; this was flushed with ca. five times the specified volume of each sample or standard before injection. A Hewlett-Packard integrator (HP3394A; Stockport, UK) was used to record the chromatograms and integrate the peak areas.

2.4. Sample preparation

2.4.1. Spiking procedure for EPI calibration and recovery

For EPI calibration and recoveries, five human serum and five water samples were each spiked with a known amount of EPI dissolved in mobile phase in the range 5–100 ng/ml (8.6–172 pmol/ml). EPI

solution (0.1 ml) that was ten-fold over strength was added to serum or water (0.9 ml) at each concentration level.

2.4.2. Spiking procedure for metabolite recovery

Solutions of EPI and its metabolites (Fig. 1) were prepared from stock master solutions in methanol. Since the concentration of each stock solution decreases slightly over time under the storage conditions used (−20°C; dark), the concentration of each was checked, after appropriate dilution, using UV spectroscopy at 480 nm (ε=12996±17 mol cm dm^{-3}). For these purposes it was assumed that the molar absorptivity of each metabolite and of EPI itself, were sensibly constant (Dr. Sardi, Pharmacia, Milan, Italy; personal communication, 1994). During development of this protocol for metabolites, blank plasma (0.9 ml) from a patient was spiked with 0.1 ml of a concentrated stock solution of EPI and its metabolites (ten-fold over-strength) to give a molar concentration equivalent to 20 ng/ml.

2.4.3. Precipitation of spiked serum and clinical samples

The precipitation solvent (0.2 ml) was slowly added to the pooled human serum or plasma (0.2 ml) in a polypropylene centrifuge tube with a conical base (volume 0.6 ml). The white precipitate was vortex-mixed for 30 s, and then centrifuged at 2000 rpm (ca. 1500 *g*) until a compact pellet and clear supernatant were obtained. Triplicate samples of serum spiked with EPI at each concentration level were prepared for validation over the concentration range 5–100 ng/ml (8.6–172 pmol/ml). One injection was made for each spiked sample. Inter-day reproducibility was tested by spiking blank patient plasma at 43 pmol/ml (25 ng/ml), and then extracting and analysing three samples, (duplicate injections) on each of five days. Spiked samples were stored at −20°C prior to analysis.

Linearity of response was checked at the beginning of each day. EPI concentrations (ng/ml) were calculated by reference to bracketting standards of EPI hydrochloride in serum at appropriate concentrations before and after each group of test samples. Metabolites were identified, where possible, by spiking with known individual standards. The amounts of reference metabolites available were limited, so, on the basis that the molar absorptivities of EOL, DD, DDL and EPI in the mobile phase at 480 nm (the excitation wavelength) are essentially identical (as noted above), and assuming that their quantum efficiencies in this solvent are similar, metabolite concentrations were estimated from the peak areas with reference to the molar concentration of EPI as a basis for calculation. Since standards for the glucuronides of EPI and EOL were not available, they were detected by incubating plasma samples with β-glucuronidase at 37°C for 4 h, to generate EPI and EOL. Control (without enzyme) and enzyme-treated samples were analysed using the HPLC system as described in Section 2.3.

Fig. 2a shows a typical chromatogram obtained from the plasma sample of a patient on day 3 of treatment. This chromatogram may be compared with that from a sample of plasma after incubation with the enzyme β-glucuronidase for 4 h (Fig. 2b). The peak at 4.33 min in the enzyme-treated sample has disappeared, whereas the EOL and EPI peaks

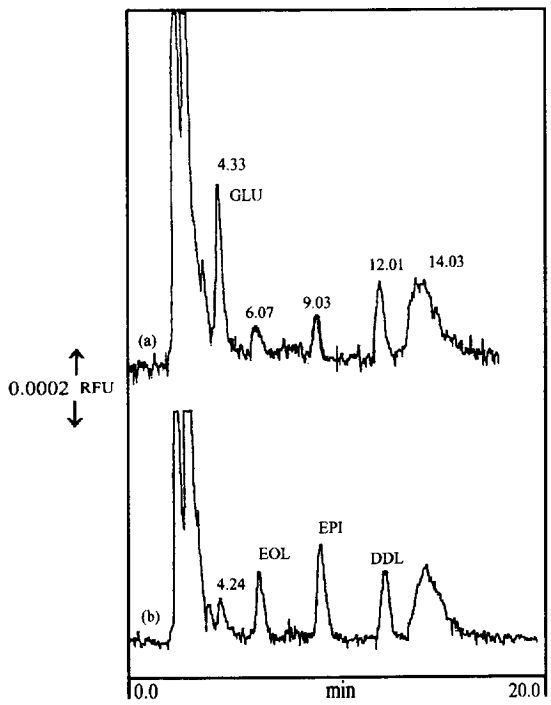


Fig. 2. Chromatograms of a clinical sample: (a) after a 4-h incubation with buffer only and (b) after a 4-h incubation with β-glucuronidase solution.

have increased by approximately 50%. The results show conclusively that the glucuronides of EPI and EOL coelute at 4.33 min. As an independent control, the peak at 12.2 min, identified as DDL, did not change significantly after incubation with this enzyme. The peak at 4.3 min is therefore assigned to glucuronide derivatives of EPI and EOL (GLU). Attempts to separate the EPI and EOL glucuronides, by for example changing the mobile phase composition, were unsuccessful. Robert and Bui [13] recently reported the separation of these metabolites using a phenyl column.

3. Results and discussion

3.1. Spiked EPI standards

Regression analysis of the data for serum compared with those for water, each spiked with EPI, demonstrated a linear response in the range 5–100 ng/ml, with good correlation between the peak area, I, and the concentration, C_{EPI}. The detector sensitivity was 0.002 relative fluorescence units, rfu (cf. manual for Gilson system). The intercept (k) and the slope (m) values were similar:

$I=5395C_{EPI} - 5759$ for human serum and $I=5636C_{EPI} - 4515$ for the aqueous standards. The correlation coefficients were 0.999 ($n=5$) in each case.

With the exception of the lower point (5 ng/ml), the overall recovery approximated to 100% (ratio of the gradients for the calibration graphs for the spiked serum and aqueous sample) and varied within the range 89–96%; R.S.D. ($n=3$) varied from 5 to 9% over this concentration range. The limit of quantitation was 8.6 pmol/ml (R.S.D.=15%). At 4.6 ng/ml the recovery values were 78, 93 and 110% (average 94%).

For convenience, the inter-day accuracy of the method was assessed at the beginning of each day by replicate injections of a 25 ng/ml aqueous standard. The acceptance criterion was ca. 8%.

The intra-day variation in the measured value for pooled human serum spiked with EPI at 25 ng/ml (43 pmol/ml) ranged from ±3.1 to ±9.2% over five days. The results of an inter-day study show that the method has a high degree of reproducibility; the R.S.D. for single measurements on a similar plasma standard (25 ng/ml) was 6.1%; the mean recovery for all standards over five days corresponded to 90% ($n=25$).

3.2. Spiked metabolite standards

Recovery of the metabolites EOL, DD and DDL at 20 ng/ml were within the range 94–104%. The R.S.D. values ranged from 3.8 to 8.4% ($n=3$). R.S.D. values for the glucuronides could not be calculated; glucuronides are known to be very soluble in neutral or slightly acidic media and recoveries are likely to be high. Fig. 3 shows a chromatogram of EPI and the available metabolites used as standards in aqueous solution.

3.3. Clinical samples

Fig. 2 shows a chromatogram of a spiked plasma sample and the detection of the glucuronide peak in a clinical sample. Fig. 4 shows a blank chromatogram of plasma taken from the patient on day 0, prior to the treatment. No endogenous interference was found corresponding to the GLU peak at 4.33 min or to the known anthracyclines in this pre-dose blank. Endogenous interference was observed as a broad peak at 16.6 min in the blank sample and in all samples examined. Its identity is unknown and it does not

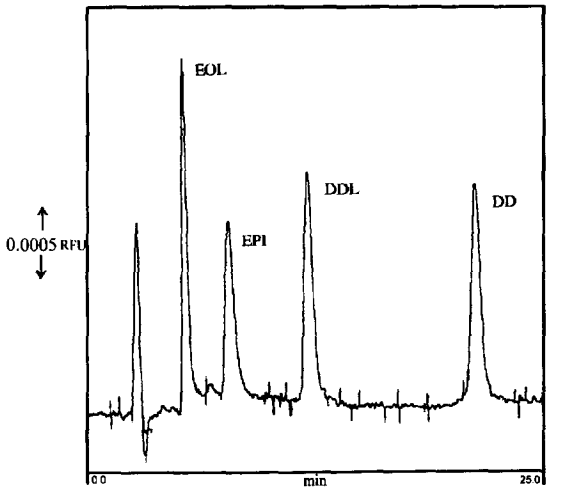


Fig. 3. Chromatogram of a standard aqueous solution containing EOL, EPI, DDL and DD, each at a concentration of 25 ng/ml.

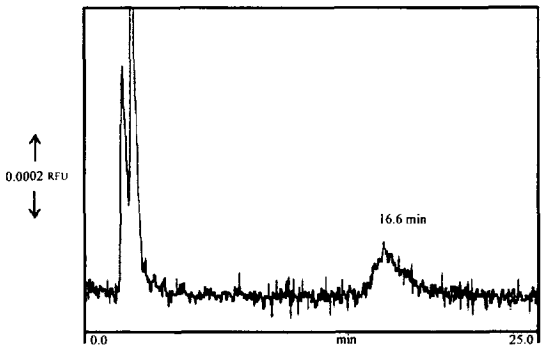


Fig. 4. Chromatogram of a pre-dose plasma sample after protein precipitation.

appear to be related to any of the common drugs taken concurrently with EPI.

Fig. 5 shows that EPI, GLU, EOL and DDL were detected on all four days of treatment for this patient. The endogenous peak at 16.6 min was detected on all five days, but the peak area did not follow any obvious trend in this case. The concentration of the parent drug, EPI, reached a maximum (~41 ng/ml) on day 2 and then decreased to ca. 13 ng/ml on day 4. The concentration of GLU rose to an estimated maximum of 38 ng/ml on the final day. The DDL and EOL concentrations reached maxima of 13 and 7 ng/ml, respectively. The aglycone DD was not detectable in any of the samples examined (LOD<3

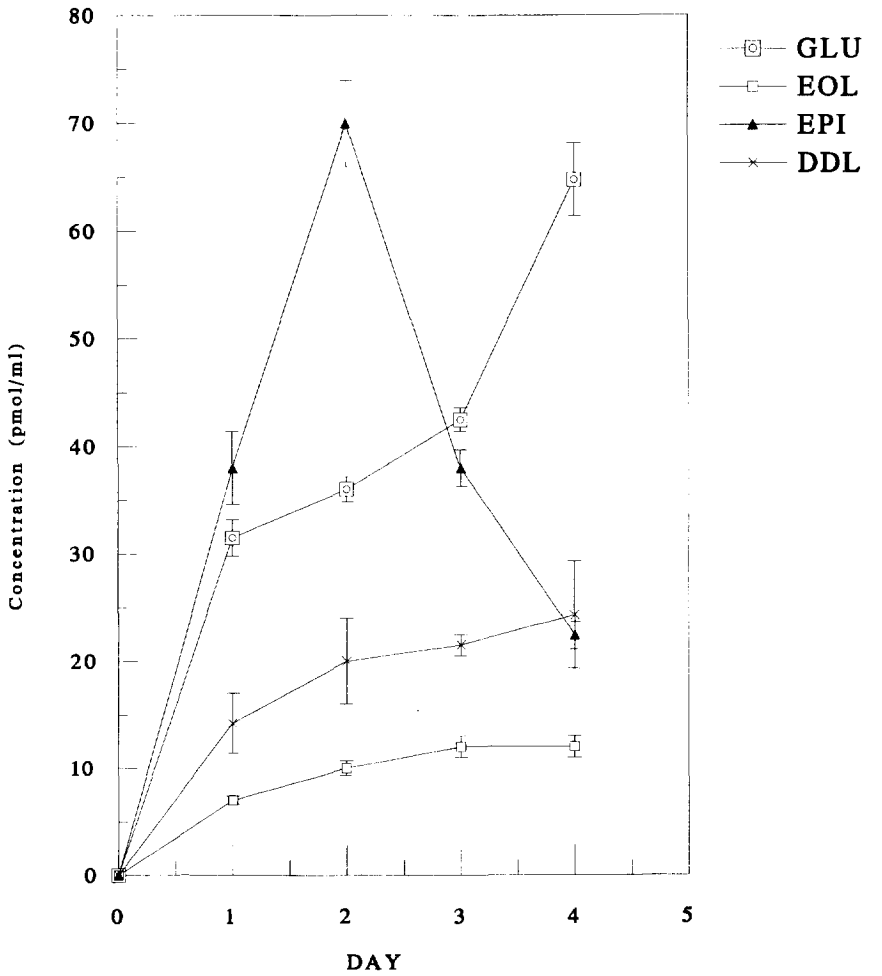


Fig. 5. Plasma EPI and metabolite concentrations during a 96-h infusion of EPI (90 mg/m^2).

ng/ml). Occasional small and unresolved peaks were not investigated further.

The use of a drug which is readily susceptible to inactivation by metabolism requires that this be taken into account when planning dosage. In particular, any individual variation in metabolism should be accounted for. This is particularly true for chronic administration, such as by protracted infusion. The case illustrated here (Fig. 5) suggests that induction of metabolism of EPI may be occurring, since there is a pronounced decrease in the observed concentration of EPI, notwithstanding the unchanged rate of infusion. Further studies therefore need to be undertaken to assess the significance and extent of this variability and the possible value of therapeutic drug monitoring. The methods described here would enable such studies to be undertaken, including observations of the metabolite profile.

A rapid and reliable method based on pre-treatment by precipitation has been shown to be applicable to clinical plasma samples of small volume (<200 μl) with low concentrations of EPI and its metabolites (<38 ng/ml). The limit of quantitation for an R.S.D. of 15% is 5 ng/ml. This analytical procedure was successfully applied to plasma samples from a patient receiving EPI chemotherapy for the first time. This protocol provides an excellent procedure for the determinatin of EPI and its metabolites at clinically relevant concentrations. However, separation of the glucuronide metabolites would require further refinement of the HPLC method.

Acknowledgments

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