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Note

High-performance liquid chromatography of iodo-doxorubicin and fluorescent metabolites in plasma samples

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The anthracyclines are an important class of anti-cancer drugs because of their broad spectrum of anti-tumour activity [1]. Daunorubicin (DNR) and doxorubicin (DOX) are the prototypes, but the search for analogues is necessary because the clinical use of DNR and DOX is hampered by side-effects, such as cumulative cardiotoxicity [2]. Furthermore, human tumours of major importance, such as lung and colon cancer, are in general not responsive to anthracyclines.

The criteria for the selection of an anthracycline analogue for clinical testing are that it should have superior preclinical antitumour activity to that of DOX, and that its cardiotoxicity should be not greater; alternatively, it should have equivalent antitumour activity and lower cardiotoxicity. One of the most extensively developed analogues is epirubicin (EPI), which is the archetype of the analogues created by configurational changes in the daunosamine. EPI is an epimer of DOX, with an equatorial instead of an axial configuration of the hydroxyl group in the C-4′-position. Otherwise it is structurally identical with DOX but this minimal change has a great influence on the pharmacokinetics and metabolism [3]. The advantage of EPI in the therapeutic index over DOX is modest, but real. The search for a "better EPI" is still going on, and another anthracycline analogue, modified at the 4′-position of the sugar moiety by replacing the hydroxyl group with an iodine atom, is undergoing phase 1 studies. The substitution of a halogen atom for the hydroxyl was planned to affect the basicity of the amino group at C-3′ and, consequently, the uptake, transport and tissue distribution of the drug (Fig. 1). At physiological pH, iodo-doxorubicin (I-DOX) is more than 95% unprotonated and much more lipophilic than DOX and EPI. This difference may partly explain the different pharmacokinetics in animals [4] and cells [5]. In preclinical studies a lack of cross-resistance to DOX-resistant cell lines and a much lower cardiotoxicity than DOX were shown [6,7].

The pharmacokinetics and metabolism of I-DOX are under phase I study. Because of the different physicochemical properties of this new anthracycline

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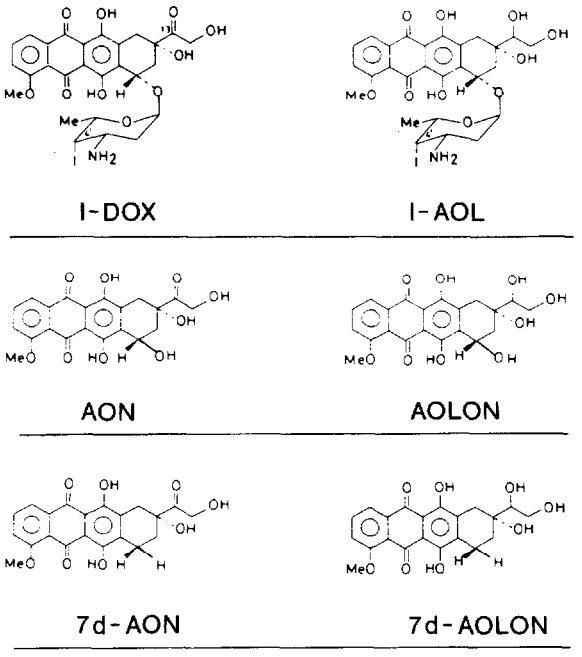

Fig. 1. Molecular structures of I-DOX and its expected metabolites. Me = Methyl.

analogue a modified analytical procedure was developed, capable of accurate determination of the unchanged drug and its known and predicted fluorescent metabolites, according to the GLP (good laboratory practice) rules. This paper describes a sensitive, accurate and simple analytical procedure for I-DOX and its metabolites (Fig. 1) in plasma. It includes an extraction with a solid–liquid procedure with high recovery for all anthracyclines, a simple isocratic high-performance liquid chromatographic (HPLC) separation with a reversed-phase column, and highly sensitive fluorescence detection.

EXPERIMENTAL

Materials

Iodo-doxorubicin (I-DOX), daunorubicin (DNR), iodo-doxorubicinol (I-AOL), doxorubicin aglycone (AON), 7-deoxydoxorubicin aglycone (7d-AON), doxorubicinol aglycone (AOLON), 7-deoxydoxorubicinol aglycone (7d-AOLON) and esorubicin (ESO) were obtained from Farmitalia Carlo Erba (Milan, Italy). Other chemicals were of analytical grade (Merck, Darmstadt, F.R.G.). Organic solvents and distilled water were of ultra-pure quality for analytical HPLC (Merck). All samples were processed in polypropylene tubes to minimize loss of anthracyclines by adsorption.

Apparatus

The HPLC system consisted of a solvent-delivery system L-6000, an auto-sampler 655 A-40, a fluorescence spectrophotometer F-1050 and a chromato-integrator D-2500 with a double disk drive (3.5 in. format, 1.44 MByte storage capacity) for data storage (Merck). The column (200 × 4.6 mm I.D.) was packed with reversed-phase C_{18} MicroSpher (3 μm particle size, ChromSep, Chrompack, Frankfurt, F.R.G.), connected to a guard column (4 × 4 mm I.D.) filled with reversed-phase material (Chrompack), which was replaced after *ca.* 50 injections.

Plasma extraction

Individual stock solutions of I-DOX and metabolites in methanol were combined to obtain an equiconcentrated stock mixture of anthracyclines. This mixture was diluted with 0.02 *M* sodium dihydrogenphosphate buffer (pH 3.0) to a range of different concentrations. Then, 100 μl of each solution and 100 μl of internal standard (50 ng DNR in 0. 02 *M* sodium dihydrogenphosphate buffer, pH 3.0) were added to 800 μl of blank plasma, resulting in a range of concentrations from 0.1 to 200 ng/ml for I-DOX and its metabolites.

Before use, Bond Elut C_{18} columns (ICT, Frankfurt, F.R.G.) were rinsed with 5 ml of methanol and 5 ml of distelled water. To process a large number of samples simultaneously a sample preparation unit with a vacuum device was used (Adsorbex SPU, Merck). Then 1 ml of blank or spiked plasma was added to the activated extraction column and purged with 4 ml of buffer (0.02 *M* sodium dihydrogenphosphate) and dried with a flow of air. The anthracyclines were eluted from the column with 4 ml of methanol–chloroform (1:1, v/v).

These samples were evaporated at 50°C under a stream of nitrogen, the residue was redissolved in 100 μl buffer and vortexed, and 50 μl were injected into the HPLC column.

Chromatographic analysis

The anthracyclines were separated and quantified by HPLC as described. The fluorescence detector was set at an excitation wavelength of 480 nm and an emission wavelength of 550 nm. The anthracyclines were eluted isocratically with a mobile phase consisting of 0.02 *M* sodium dihydrogenphosphate–acetonitrile (3:2, v/v) (pH 3.0). The flow-rate was 0.8 ml/min.

Quantitative analysis

A calibration curve was established by analysing blank plasma samples spiked with known amounts of the parent drug, the metabolites and DNR as internal standard. The area under the peak and the peak heights were calculated by the integrator. Loss of anthracyclines during the extraction procedure was compensated by use of the internal standard procedure. All samples were processed in duplicate.

Quality control

Calibration curves were set twice a week a total of six times (always in duplicate), and once a six-fold analysis of full calibration curves was done. The recovery of DNR was checked 240 times during the development of the assay.

RESULTS

Chromatograms

Injection of the anthracyclines (I-DOX, DNR, and metabolites) into the HPLC system resulted in a complete separation of all metabolites (Fig. 2) within 12 min. Differences in peak heights arise from differences in retention times, and the fact that the different anthracyclines have their maximum for emission at different wavelengths whereas the detector emission wavelength is fixed.

Injection of extracted plasma samples from a patient after I-DOX administration resulted in the other chromatogram shown in Fig. 2. There is an extra peak present. If dehalogenation takes place the formation of esorubicin (ESO) can be expected. The peak at the front of the chromatogramm represents fluorescent material co-extracted from the plasma.

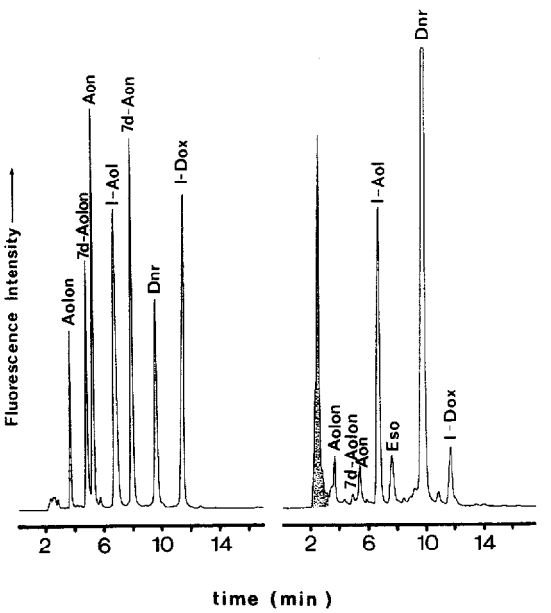

Fig. 2. (Left) Complete separation of I-DOX and its metabolites (artificial metabolite mix); detector setting at the highest sensitivity level. (Right) Separation of I-DOX and its metabolites after extraction of a patient plasma sample (after I-DOX administration); detector setting at the highest sensitivity level. The patient received 40 mg I-DOX i.v.; a plasma sample was taken after 1 h. The hatched area represents polar fluorescent compounds present in each plasma sample.

TABLE I

RECOVERIES OF I-DOX AND ITS METABOLITES

Compound	Recovery (mean + S.D., $n=6$) (%)				
	1 ng/ml	10 ng/ml	50 ng/ml	100 ng/ml	200 ng/ml
I-DOX	98±7	100±3	95±7	100±4	87±5
I-AOL	96±5	97±3	89±4	89±6	75±5
AON	95±6	93±5	90±5	85±8	80±3
7d-AON	98±5	100±4	97±6	87±5	85±3
AOLON	93±6	94±5	93±6	87±4	79±2
7d-AOLON	96±4	97±2	88±7	75±9	70±10

Detector linearity and detection limit

The detector response was found to be linear for injections of absolute amounts of DNR in the range 0.1–50 ng. The detection limit at a signal-to-noise ratio of 2 ranged from 0.05 ng/ml for AON, 7d-AON and AOLON, to 0.1 ng/ml for I-DOX, I-AOL and 7d-AOLON.

Recovery

Recoveries of the anthracyclines from spiked plasma were calculated by comparing the peak heights in the chromatograms of extracted samples with those of standards with identical concentrations in buffer. Recoveries ranged from 70% for 7d-AOLON to 100% for I-DOX (Table I). The evaporation procedure (heat-

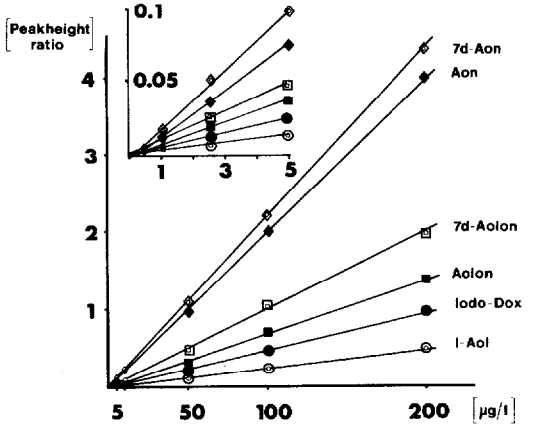

Fig. 3. Linearity of the calibration curves (mean of within-day values, $n=6$) of I-DOX and metabolites in plasma with DNR as internal standard.

TABLE II

WITHIN-DAY AND BETWEEN-DAY PRECISION OF CONCENTRATIONS FROM CALIBRATION CURVES

Compound	Within-day ($n=6$) S.D. of concentrations (%)				Between-day ($n=6$) S.D. of concentrations (%)		
	200 ng/ml	100 ng/ml	5 ng/ml	0.5 ng	100 ng/ml	5 ng/ml	0.5 ng
I-DOX	4	5	5	6	4	4	7
I-AOL	4	5	5	11	4	6	12
AON	3	3	4	15	3	5	10
7d-AON	3	2	5	4	3	5	6
AOLON	3	3	5	5	3	6	7
7d-AOLON	5	6	6	8	7	5	8

ing the samples to 50°C) has no adverse influence on the recovery: the anthracyclines were stable during the procedure. The recovery of DNR was 88 ± 2%.

Calibration lines, within-day and between-day variation of the slopes

In Fig. 3, mean values of the within-day peak-height ratios of I-DOX and its metabolites to the internal standard (DNR) are plotted against the concentration in plasma (ng/ml). Calibration lines were calculated by the least-squared method. Such plots were linear for all compounds, with coefficients of regression (r^2) better than 0.99. The within-day ($n=6$) and between-day ($n=6$) variations of the slopes were 2–5% and 3–13% respectively.

Intra- and inter-assay precision

The within-day precision of the assay was established by the analysis of plasma samples spiked with I-DOX and its metabolites at four different concentrations, repeated six times. The between-day variance was calculated from the daily mean of duplicate analyses of plasma samples containing I-DOX and its metabolites at three different concentrations, analysed over six consecutive days. All values are shown in Table II.

DISCUSSION

The described HPLC assay for iodo-doxorubicin and its metabolites allows the accurate quantitative analysis of plasma samples, with great improvements in detection limit, recovery rate and precision. The general assay procedure itself is similar to the previously described assay for epirubicin [8], but it is not so laborious owing to the use of a sample preparation unit (SPU) with a vacuum device

to allow the simultaneous processing of 24 samples. Additionally, the within-day and between-day precision have been significantly improved compared with the previous equivalent data [8], a fact related to the use of the SPU. The mobile phase contained more acetonitrile because these anthracyclines are less polar. Another major aspect is the lower detection limit, now in the picogram region, a fact related to the new detector being used, which allows a longer monitoring time during pharmacokinetic studies and consequently more reliable calculations of major pharmacokinetic data. The recovery rates for nearly all anthracyclines are better than the previous values [8]; an improvement of 15–20% was observed, because of the better extraction cartridges and columns and modifications to the eluent.

The special feature of the assay developed for EPI was the high extraction efficiency for the very polar metabolites, because the previously used organic solvent extraction procedures did not allow quantitative recoveries of the glucuronides of EPI and epirubicinol.

In the case of I-DOX, glucuronidation seems to be impossible at the 4′-position of the sugar moiety because of the presence of the halogen atom. I-DOX is much more lipophilic than DOX. Metabolites that can be expected are the aglycones and 7-deoxyaglycones in the case of cleavage of the sugar moiety. Additionally, via carbonyl reduction at C-13 by aldoketoreductase, iodo-doxorubicinol should be expected as a major metabolite.

One more metabolite can be generated under extreme conditions. From the gut microflora it is known that it is very active in reductive reactions [9]. Clearly, ingested compounds that are poorly absorbed from the gut will stand the greatest chance of undergoing metabolism through the intestinal flora, although a large number of compounds gain entry to the gut via biliary secretion. The anthracyclines are preferentically excreted via biliary secretion. If reductive dehalogenation takes place in the gut, esorubicin may be generated as a metabolite. This cytostatic drug is under clinical investigation [10].

As can be seen in the chromatograms, all these metabolites ca be quantified accurately with the described HPLC procedure, allowing pharmacokinetic and metabolism studies within different clinical studies. The first results of such studies will be published elsewhere [11].

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