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HPLC analysis of doxorubicin, epirubicin and fluorescent metabolites in biological fluids*

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Summary. A specific, sensitive and reliable HPLC method for the determination of doxorubicin (DX), epirubicin (epiDX) and their known fluorescent metabolites [doxorubicinol (DXol), epirubicinol (epiDXol) 7-deoxy-adriamycinone (metabolite C), adriamycinone (metabolite D), 7-deoxy-13-dihydro-adriamycinone (metabolite E), 13-dihydro-adriamycinone (metabolite F), 4'-*O*-β-D-glucuronyl-4'-epiDX (metabolite G) and 4'-*O*-β-D-glucuronly 13-dihydro-4'-epiDX (metabolite H)] in biological fluids (human plasma, bile and urine) has been developed and tested. Plasma samples were solid-phase-extracted (C_{18}-bonded silica cartridges). Urine and bile samples were injected directly after the addition of the internal standard and dilution with 0.3 *M* phosphoric acid. Complete separation of unchanged drugs and metabolites was achieved with a mobile phase consisting of 75.6% 10 m*M* KH_2PO_4 and 24.4% CH_3CN (the pH of the solution was adjusted to 4.3 with 0.03 *M* H_3PO_4) at a flow rate of 1.5 ml/min. For the analyses we used a cyanopropyl chromatographic column (25 cm × 4.6 mm i.d.; particle size 5 μm) and fluorescence detection with excitation wavelength set at 470 nm and emission at 580 nm. Sensitivity was better than 0.3 ng/ml for all substances analysed. The mean absolute recovery of unchanged drugs and metabolites was between 88.3% (metabolite E) and 98.92% (metabolite G). Intra- and interassay precisions (plasma samples) were better than 10.6% and 13.0%.

Introduction

Doxorubicin (DX) is an anthracycline antibiotic widely used in the treatment of neoplastic diseases in man; the new derivative epirubicin (epiDX) differs from DX in the epimerization of the OH group in position 4' of the amino sugar moiety [1]. Preclinical studies in animal models and extensive clinical trials have proven that this compound is less toxic than DX at similar and equipotent therapeutic doses [13].

We studied the comparative pharmacokinetics and metabolism of DX and epiDX in a group of eight cancer patients who received 60 mg/m^2 of both drugs by i.v. bolus injection according to a balanced cross-over design [6]. A new analytical procedure was therefore developed, capable of accurately determining unchanged drugs and known fluorescent metabolites in the concentration range typically observed in biological fluids up to 168 h after drug administration.

Several HPLC assays for DX and epiDX can be found in the literature [2–4, 7–10, 12]. When anthracycline metabolites were detected, no specific calibration was generally performed, thus assuming similar analytical behaviour for unchanged drug and biotransformation products. Moreover, the commonly used organic solvent extraction of plasma samples followed by counterextraction with 0.03 *M* phosphoric acid leads to the extensive loss of the glucuronic acid conjugates, which are not efficiently extracted in the organic phase, as well as of anthracycline aglycones, which are not back-extracted in the acidic phase due to their low solubility in aqueous media.

Solid-phase extraction with C_{18} reversed-phase cartridges followed by HPLC separation and fluorimetric detection allows the simultaneous quantitative determination of DX, epiDX and known fluorescent metabolites in biological samples [5, 11]. Here we report the experimental details and the validation data of such an assay procedure.

Materials and methods

Materials. Doxorubicin (DX), epirubicin (epiDX), doxorubicinol (DXol), epirubicinol (epiDXol), 7-deoxy-adriamycinone (aglycone C), adriamycinone (aglycone D), 7-deoxy-13-dihydro-adriamycinone (aglycone E), 13-dihydro-adriamycinone (aglycone F), 4'-*O*-β-D-glucuronyl-4'-epiDX (glucuronide G) and 4'-*O*-β-D-glucuronyl-13-dihydro-4'-epiDX (glucuronide H), used as reference standards, and daunorubicin (IS), used as internal standard, were obtained from Farmitalia R&D Laboratories.

The reagents were of analytical grade (Farmitalia – Carlo Erba, Milan) and were used without further purification; laboratory grade distilled water was purified with removal of residual ions and organic impurities with a MILLI-Q Water System (Millipore S. A., Molsheim, France) and was filtered through a 0.25-μm membrane filter.

All the glassware used had to be previously deactivated by treatment with 10% dimethyldichlorosilane in anhydrous toluene, followed by absolute methanol washing.

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This process prevents drug complexation and degradation on the active catalytic centres (free SiOH groups) of the glassware.

Apparatus. Chromatographic analysis was performed with a Varian model 5000 liquid chromatograph fitted with a Perkin-Elmer 650-10LC fluorescence detector (excitation wavelength, 470 nm; emission, 580 nm; slit width, 20 nm) and a Supelcosil LC-CN chromatographic column (25 cm × 4.6 mm i.d. particle size 5 μm).

Peak area determination and internal standard calculations were carried out with a Varian Vista 401 data system. Chromatographic peaks of 1000 area counts were recorded with signal-to-noise ratios of 3–7.5 and were considered the detection limit of the system.

Plasma extraction. We added 10–100 μl internal standard stock solution (daunomicyn hydrochloride, 1 μm/ml in distilled water), 1 ml 10 m*M* phosphate buffer (pH 8) containing 0.6 μ*M* tetrabutylammonium bromide, and 1 ml methanol to plasma samples (1 ml). The solution was filtered through a Sep-Pak C_{18} cartridge (Waters Assoc., Milford, Mass) previously washed with methanol (3 ml) and 10 m*M* phosphate buffer – methanol 3:1 (3 ml). The cartridge was eluted first with 3 ml water – methanol 3:1 (discharged), then with 3 ml 0.03 *M* phosphoric acid in methanol. This solution, after the addition of 100 μl 0.1 *M* KH_2PO_4, was evaporated in a Buckler vortex evaporator under vacuum (50-5 torr) at 25° C. The residue (100–400 μl) was analysed by HPLC (10- to 100-μl injections; the amount injected was chosen so as to maintain the peak area counts in the range 1000–300000). To maximize method sensitivity and linearity, the amount of plasma extracted (and the amount of internal standard added to the solution) depended on the expected sample concentration.

Urine and bile. Urine and bile samples (1 ml) were injected directly onto the chromatographic system after addition of the internal standard and dilution with 1 ml distilled water and 1 ml 0.3 *M* phosphoric acid.

Chromatographic analysis. During assay development, components were eluted isocratically with a mobile phase consisting of phosphate buffer – acetonitrile mixtures (pH range: 2–4.5; percentage of organic modifier: 24%–32%) at a flow rate of 1–1.5 ml/min.

Routine analysis in the application laboratory was usually performed with a mobile phase consisting of 75.6% of 10 m*M* KH_2PO_4 and 24.4% CH_3CN, adjusted to pH 4.3 with 0.03 *M* H_3PO_4, at a flow rate of 1.5 ml/min. As relative retention times of free drugs and metabolites are sensitive to variations in chromatographic conditions, the composition of the mobile phase was sometimes slightly adjusted (pH and/or percentage of organic modifier) to compensate for variations in column efficiency.

Identification of free drugs and metabolites in biological fluids was achieved by comparison with the authentic specimens obtained from the Farmitalia R&D laboratories. Retention times under various chromatographic conditions (pH and percentage of the organic modifier) and the identity of fluorescence spectra recorded after stopping the mobile phase flow during peak elution constituted the comparison criteria.

Additional identification of the glucuronic acid conjugates G and H was achieved by incubating samples (0.5 ml) with a known amount of internal standard, β-glucuronidase and 0.1 *M* phosphate buffer (0.1 ml) at pH 6.8, 37° C for 4 h. As a control, an equal amount of the sample was incubated in the same conditions but without β-glucuronidase. HPLC assay of the two samples shows the disappearance of conjugated metabolites and the simultaneous increase of the epiDX and epiDXol peaks in the enzyme-treated sample.

Quantitative analysis. Calibration of the analytical method was carried out by analysing, as described above, blood bank plasma samples (or urine and bile samples) spiked with known amounts of free drugs, metabolites and daunomycin hydrochloride as internal standard. Calibration factors (Rf) were then computed as

$$Rf_x = C_x.A_{is}/C_{is}.A_x$$

where C_x is the known concentration of compound x, C_{is} is the known concentration of the internal standard (daunomycin hydrochloride), and A_x and A_{is} are the areas under chromatogrpahic peaks relative to compound x and internal standard respectively. Each calibration was carried out in triplicate.

Quality control. In the application phase, a complete calibration run was performed each time a patient was treated with epiDX or DX in the cross-over study [6]. Day-to-day quality control was also performed by randomly submitting blank and spiked plasma samples.

The identity of retention times was controlled daily by adding known amounts of authentic specimens to the unknown mixture.

Results

Chromatograms and column efficiency

Figure 1 shows the dependency of capacity factors k' of unchanged drugs and metabolites on the pH of the mobile phase. The number of theoretical plates of the column recorded in these experimental conditions was between 9702 and 19146.

Plasma, urine and bile samples obtained before treatment were free of interfering fluorescent compounds. Figure 2 shows typical chromatograms relative to routine analysis of plasma samples obtained from patients treated with epiDX and DX.

Detector linearity and minimal detectable concentration

The detector response was found to be linear for injections of absolute amounts of daunomycin hydrocloride at least in the range 0.108 ng (area counts: 956) to 53.7 ng (area counts: 474 356). The actual amount of sample injected in both calibration and analytical runs was therefore selected in order to obtain peak area counts in the range 1000–300000; chromatographic peaks of 1000 area counts were considered to represent the lowest detectable quantity. This coresponds to a sensitivity limit of 0.255 ng/ml for epiDX, 0.197 ng/ml for epiDXol, 0.255 ng/ml for DX, 0.197 ng/ml for DXol, 0.274 ng/ml for metabolite C, 0.186 ng/ml for metabolite D, 0.266 ng/ml for metabolite

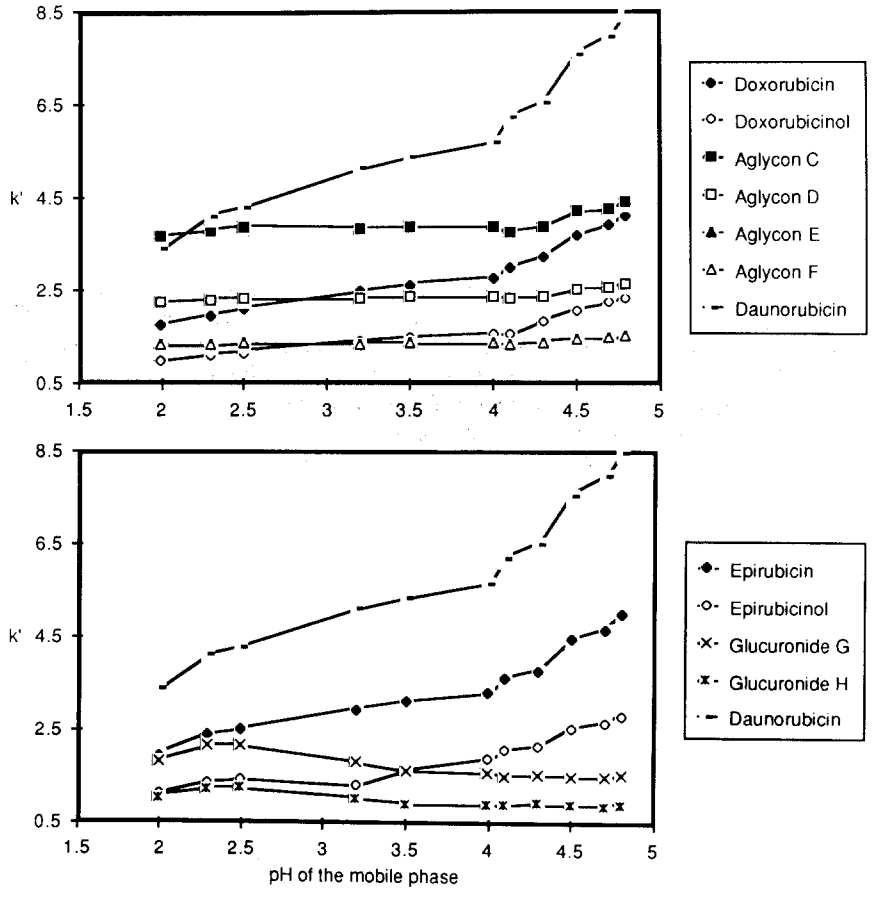

Fig. 1. Dependency of capacity factors k' of doxorubicin, epirubicin and metabolites on the pH of the mobile phase

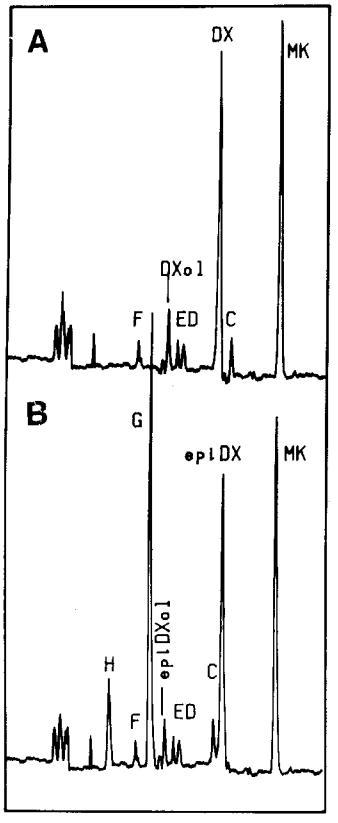

Fig. 2. HPLC analysis of plasma samples obtained from patients treated with doxorubicin (chromatogram A) and epirubicin (chromatogram B). (*C, D, E, F*, aglycons; G, H, epiDX and epiDXol glucuronides; *MK*, internal standard)

Table 1. Recovery

Compound	Recovery (%)			No.	C.V.
	mean	min.	max		
epiDX	94.87	82.69	109.95	12	8.37
epiDXol	97.06	80.59	105.41	12	7.45
Aglycone C	89.39	77.52	108.20	12	10.46
Aglycone D	91.88	81.80	105.30	12	7.16
Aglycone E	88.30	73.97	96.50	12	7.27
Aglycone F	96.11	85.31	120.07	12	9.74
Glucuronide G	98.92	86.88	104.90	12	6.03
Glucuronide H	98.34	89.80	110.99	12	5.82
DX	94.26	84.45	103.85	12	7.22
DXol	98.30	89.89	105.20	12	5.50
Internal standard	94.67	87.30	104.99	9	6.64

No., Number of experiments; CV, coefficient of variation

E, 0.179 ng/ml metabolite F, 0.23 ng/ml for metabolite G and 0.23 ng/ml for metabolite H (plasma samples, 2-ml extractions).

Recovery

Recovery data for free drugs, metabolites and internal standard are reported in Table 1. Absolute recovery was always better than 74%.

Intra- and interassay precision

Reference factors and intra- and interassay precision data are reported in Table 2. These data were deter-

Table 2. Calibration factors (Rf) and intra- and interassay precision

Compound	Plasma			Bile			Urine		
	Rf	Intraassay	Interassay	Rf	Intraassay	Interassay	Rf	Intraassay	Interassay
epiDX	1.19	7.74	8.02	1.04	6.57	7.17	1.06	5.59	7.22
epiDXol	0.91	6.28	7.47	1.00	5.25	6.91	1.01	6.24	10.38
Aglycone C	1.27	6.38	8.02	1.37	8.39	11.28	1.30	6.29	14.47
Aglycone D	0.86	6.30	8.26	1.01	4.56	9.77	0.99	4.39	9.81
Aglycone E	1.23	7.55	11.19	1.29	3.62	12.56	1.30	9.13	12.01
Aglycone F	0.83	5.99	9.74	0.91	4.59	4.64	1.07	3.80	11.68
Glucuronide G	1.06	7.85	9.18	1.17	4.47	5.02	1.21	6.00	10.37
Glucuronide H	1.06	10.58	13.20	1.12	8.95	9.14	1.02	8.41	9.40
DX	1.18	6.96	9.14	1.04	5.00	6.09	1.06	7.25	7.58
DXol	0.91	5.64	8.19	1.03	4.48	10.97	1.03	6.70	7.09

Rf, Calibration factor; intraassay, mean intraassay coefficient of variation (multiple Rf determinations in a single sample); interassay, interassay coefficient of variation on multiple Rf determinations

mined at nine concentration levels in the ranges 0.773–973.35 ng/ml (epiDX), 0.951–392.81 ng/ml (epiDXol), 0.582–3512.0 ng/ml (DX), 0.767–3078.8 ng/ml (DXol), 0.914–511.12 ng/ml (metabolite C), 0.777–443.1 ng/ml (metabolite D), 3.669–283.3 ng/ml (metabolite E), 5.393–351.7 ng/ml (metabolite F), 3.984–415.7 ng/ml (metabolite G), and 0.415–43.31 ng/ml (metabolite H).

Discussion

This analytical procedure differs in two ways from the earlier assays described by ourselves and other authors, i.e. extraction procedure and chromatographic conditions. Furthermore, calibration was performed for all the substances analysed, without assuming similar extraction and fluorescence efficiency for all compounds.

The use of solid-phase extraction allows the separation of unchanged drugs and metabolites from plasma samples and their subsequent recovery in higher yields. The efficiency of the extraction procedure is also shown by the relatively small difference in Rf values determined for plasma samples and for urine and bile samples. Urine and bile samples were not extracted, but were directly injected into the chromatographic apparatus. Larger differences in the Rf values would have been generated had there been a selective loss of compounds during the extraction procedure.

The addition of the ion-pairing reagent tetra *n*-butyl ammonium bromide (TBA) to the plasma samples leads to a dramatic improvement in the recovery of the G and H glucuronides. In early experiments carried out in the absence of TBA, a significant amount of G and H was eluted from the cartridge during the plasma extraction, and in the collection step only 40%–60% of the glucuronides were recovered.

C and E deoxyaglycons are characterized by particularly low solubility in water, methanol and acetonitrile. This behaviour is reflected by less reproducible extraction of these metabolites if they are present in relatively high concentrations. This problem, however, does not affect the overall reliability of the assay, since metabolites C and E are present in blood, bile or urine of patients at concentration levels that can be reliably determined by this method.

In the current study, the complete separation and simultaneous determination of unchanged drugs and metabolites was achieved with a cyanopropyl bonded phase chromatographic column. A C_{18} reversed-phase column could also be used. Slightly better resolution of closely eluted peaks could be achieved by this means but at the cost of longer retention times, broader peaks and consequently less sensitivity, owing to a less favourable signal-to-noise ratio.

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