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IMPROVED METHOD FOR THE DETERMINATION OF 4′-EPIDOXORUBICIN AND SEVEN METABOLITES IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

4′-Epidoxorubicin, its seven metabolites and doxorubicin, as internal standard, were efficiently extracted from plasma using C_{18} Sep-Pak cartridges. The recoveries ranged from 58% for doxorubicin aglycone up to 98% for 4′-epidoxorubicin glucuronide. The anthracyclines were separated by reversed-phase high-performance liquid chromatography within 9 min and analysed by fluorescence. The assay was sensitive to $3 \cdot 10^{-10}$ *M* for the glucuronides up to $12 \cdot 10^{-10}$ *M* for 7-deoxydoxorubicin aglycone. The peak-height ratio of the fluorescence intensities of the anthracyclines versus doxorubicin showed a linear correlation with the concentration from the detection limit up to $2.5 \cdot 10^{-7}$ *M* (correlation coefficient $r^2 > 0.99$). Within-day and between-day precision of the assay were in the ranges 2–14% ($n=6$) and 2–11% ($n=6$), respectively.

INTRODUCTION

Anthracyclines represent an important class of compounds used in cancer chemotherapy. 4′-Epidoxorubicin (E), a relatively new anthracycline [1], is presently under investigation in clinical phase III studies. Its structure differs from that of doxorubicin (A) in the orientation of the 4′-hydroxy group in the daunosamine moiety. E has a spectrum of anti-tumour activity comparable with that of A, but with lower cardiotoxicity [2]. The structures of the metabolites of E found in urine (Eol-glu, E-glu and Eol) and in plasma (Eol-glu, E-glu, Eol, Aon, Aolon, 7d-Aon and 7d-Aolon) are shown in Fig. 1.

Pharmacokinetic studies of E and its metabolites require a sensitive and reproducible technique to quantify these compounds in biological fluids. However, low recoveries from plasma have been reported [3] for the 4′-glucuronides, and high-performance liquid chromatographic (HPLC) procedures [4–14] were not able to separate all metabolites of E and A.

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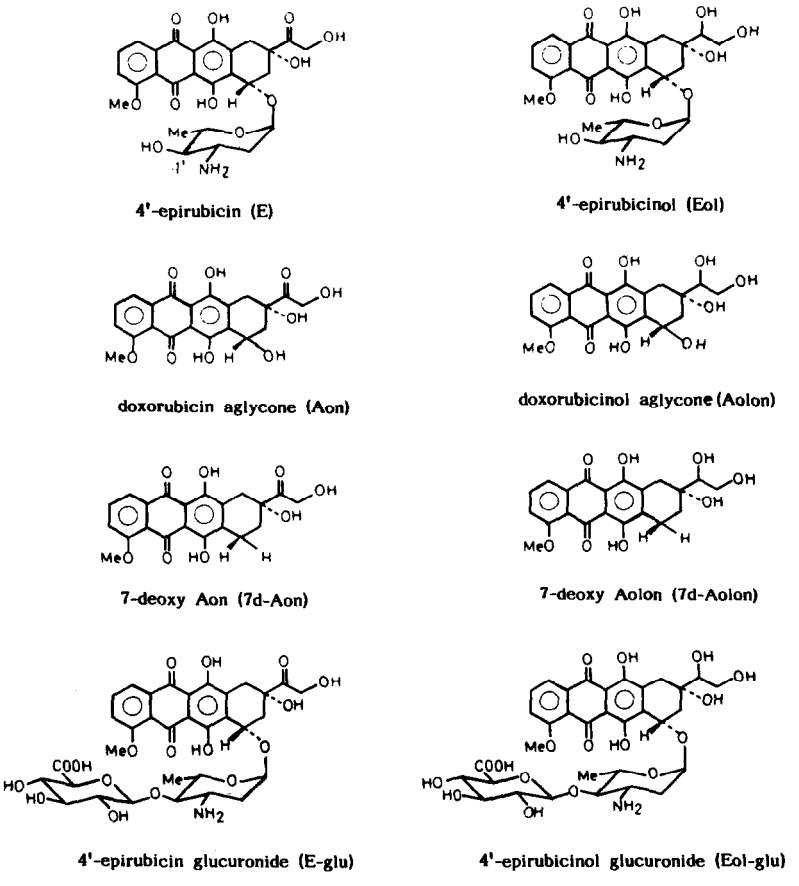

Fig. 1. Structural formulae of 4'-epidoxorubicin and its metabolites.

This paper describes a more sensitive, accurate and universal analytical procedure for E and all its known metabolites in plasma. It includes extraction with high recovery for all metabolites, a simple isocratic HPLC separation and highly sensitive fluorescence detection.

EXPERIMENTAL

A, E and its metabolites were kindly provided by Farmitalia Carlo Erba (Milan, Italy). Acetonitrile was of analytical grade (Merck, Amsterdam, The Netherlands). Buffer and acetonitrile were purified by membrane filters from Sartorius (Breukelen, The Netherlands; Nos. 11107 and 11607, respectively).

Individual stock solutions of E and metabolites in methanol were combined to obtain an equimolar mixture of anthracyclines. This mixture was diluted with a buffer, 0.019 *M* sodium dihydrogen phosphate pH 4–acetonitrile (9:1, v/v), to a range of different concentrations. Then, 50 μl of this solution and 50 μl of internal standard (compound A) in buffer were added to 900 μl of blank plasma, resulting in a range of concentrations from $5 \cdot 10^{-10}$ to $2.5 \cdot 10^{-7}$ *M* for E and metabolites.

Two internal standard concentrations were used: $1.25 \cdot 10^{-8}$ *M* for E and its metabolites in the concentration range from $5 \cdot 10^{-10}$ to $2.5 \cdot 10^{-8}$ *M*, and $1.25 \cdot 10^{-7}$ *M* in the concentration range from $2.5 \cdot 10^{-8}$ to $2.5 \cdot 10^{-7}$ *M*. In each solution Eol-glu was present at one sixth of the concentration of the other compounds.

Before use, C_{18} Sep-Pak cartridges (Waters, Etten-Leur, The Netherlands) were successively rinsed with 5 ml of methanol, 5 ml of distilled water and 5 ml of buffer. Then, 1 ml of blank or spiked plasma was added and washed with 2 ml of buffer. The anthracyclines were eluted from the cartridge with 4 ml of methanol–chloroform (3:1, v/v). After evaporation of the solvents at 50°C under a stream of nitrogen, the residue was redissolved in 50 μl of buffer, of which 30 μl were injected onto the HPLC column.

A, E and its seven metabolites were separated and quantified by an HPLC system consisting of a WISP 710B injection system, a Model 6000A solvent-delivery system and a data module with system controller 720 (Waters). This system was provided with a stainless-steel HPLC column (100×4.6 mm I.D., 3 μm CP MicroSpher; Chrompack, Middelburg, The Netherlands) including a guard-column (4×4 mm, 5-μm LiChrosorb RP-18; Merck) and an F1000 fluorescence detector (excitation wavelength 480 nm, emission wavelength 580 nm) from Merck-Hitachi (Amsterdam, The Netherlands). Elution was under isocratic conditions with 0.019 *M* sodium dihydrogen phosphate pH 4–acetonitrile (2.25:1, v/v) at a flow-rate of 1.0 ml/min.

RESULTS AND DISCUSSION

The eluents produced after the introduction of the spiked plasma sample into the Sep-Pak cartridge were spectrofluorometrically analysed for the presence of anthracyclines. No anthracyclines were detectable after the purge with buffer, whereas 4 ml of methanol–chloroform appeared to be sufficient to elute most of the anthracyclines of interest. Injection of the recovered anthracyclines into the HPLC system resulted in a complete separation of all metabolites within 9 min (Fig. 2b). Injection of a similarly treated blank plasma sample resulted in the chromatogram shown in Fig. 2a. More contaminations were observed in the chromatogram when either acetonitrile was omitted in the buffer or more chloroform [15] was used for the final elution from the Sep-Pak cartridge. Conversely, absence of chloroform produced lower recoveries, particularly in the case of the least polar 7-deoxy compounds. In the absence of chloroform these metabolites tend to form self-association products, which appear to be immobilized on reversed-phase material [3]. Our elution step inhibits formation of these products, resulting in high recoveries without introducing undesirable plasma components.

The chromatogram of an equimolar mixture of anthracyclines is shown in Fig. 2b. Differences in peak heights arise from differences in recovery, retention times and/or fluorescence quantum yields of the individual compounds. Fig. 2c shows a chromatogram of plasma from a patient 9 h after administration of 50 mg E per m^2. The detection limit at a signal-to-noise ratio of 2 ranged from $1.2 \cdot 10^{-10}$ to $12 \cdot 10^{-10}$ *M*, with the lowest values for the more polar anthracyclines (Table I).

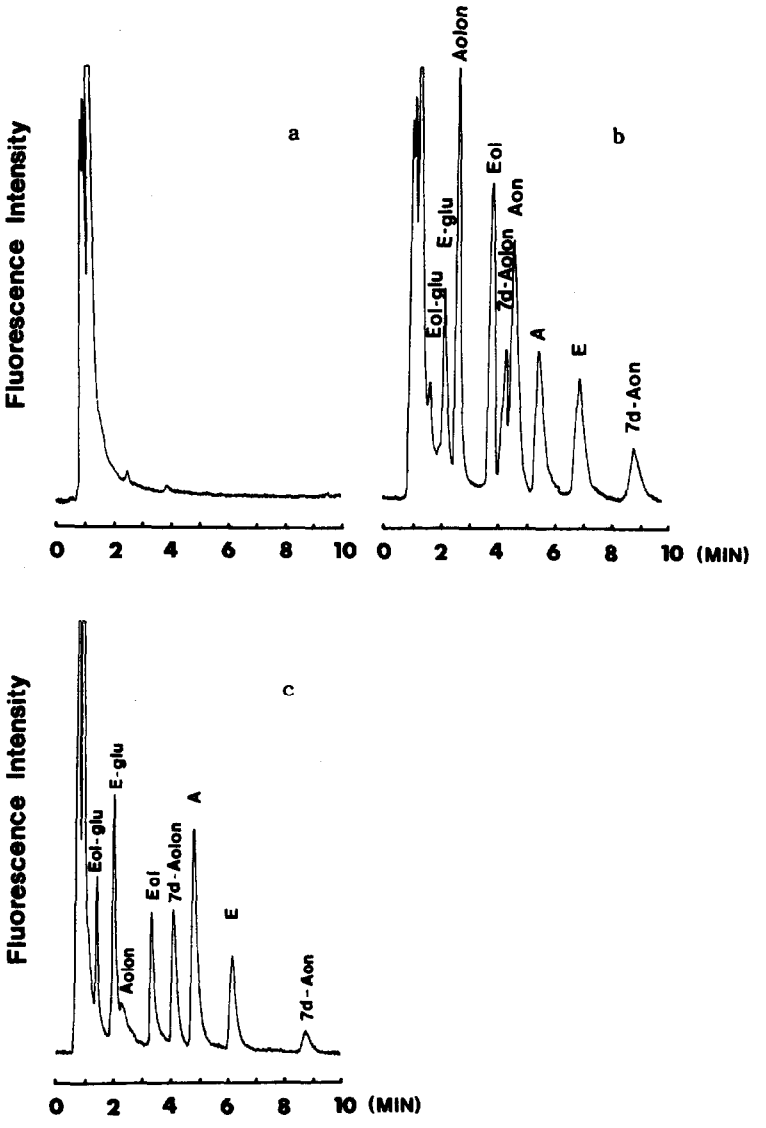

Fig. 2. Typical chromatograms of (a) extracted blank plasma, (b) plasma spiked with equimolar amounts of E and metabolites ($1.25 \cdot 10^{-8}$ *M*; the concentration of Eol-glu is $2.1 \cdot 10^{-9}$ *M*) and A ($1.25 \cdot 10^{-8}$ *M*) as internal standard, and (c) plasma of a patient 9 h after administration of 50 mg E per m^2 with A as internal standard. Detector setting: sensitivity, 20; time constant, 1; excitation wavelength, 480 nm; emission wavelength, 580 nm.

The recoveries of the anthracyclines from spiked plasma samples were calculated by comparing peak heights in the chromatograms of extracted samples with those of standards with identical concentrations in buffer. Recoveries ranged from 58 to 98%, increased with increasing concentration and varied between metabolites (Table II). The highest values were found for the glucuronides (the most polar compounds of all metabolites studied). These high yields (68–98%) are a

TABLE I

DETECTION LIMITS OF A, E AND METABOLITES

Compound	Detection limit ($\times 10^{10}$ M)
Eol-glu	3
E-glu	3
Aolon	1.2
Eol	2.4
7d-Aolon	6
Aon	2.4
A	2.4
E	3.6
7d-Aon	12

major improvement over the yields from liquid–liquid extraction reported earlier [3], but of the same magnitude as those from other solid-phase extraction procedures [15]. The least polar 7-deoxy compounds are recovered in the range 63–76%.

In Fig. 3, mean values of the within-day peak-height ratios of E and its metabolites to the internal standard A are plotted against the concentration in plasma. Calibration lines were calculated by the least-squares method. These plots were linear for all compounds from $5 \cdot 10^{-10}$ up to $2.5 \cdot 10^{-7}$ M, with coefficients of regression (r^2) better than 0.998. At concentrations above $2.5 \cdot 10^{-7}$ M, deviation from linearity may be expected [3] for the 7-deoxyaglycones because of their tendency to associate.

Table III shows the within-day ($n=6$) and between-day ($n=6$) variation of the slopes of the calibration lines for each metabolite. These data clearly indicate

TABLE II

RECOVERIES OF A, E AND ITS METABOLITES

Compound	Recovery ± C.V. ($n=6$) (%)		
	$0.5 \cdot 10^{-8}$ M	$2.5 \cdot 10^{-8}$ M	$12.5 \cdot 10^{-8}$ M
Eol-glu*	68 ± 11	80 ± 4	97 ± 6
E-glu	79 ± 3	90 ± 2	98 ± 5
Aolon	74 ± 4	76 ± 2	78 ± 5
Eol	62 ± 3	71 ± 9	90 ± 6
7d-Aolon	65 ± 8	68 ± 9	75 ± 4
Aon	68 ± 4	58 ± 6	77 ± 5
A		62 ± 8**	
E	64 ± 7	61 ± 6	82 ± 5
7d-Aon	64 ± 8	68 ± 3	76 ± 6

*The Eol-glu concentration is one sixth of the stated values.
**$1.25 \cdot 10^{-8}$ M.

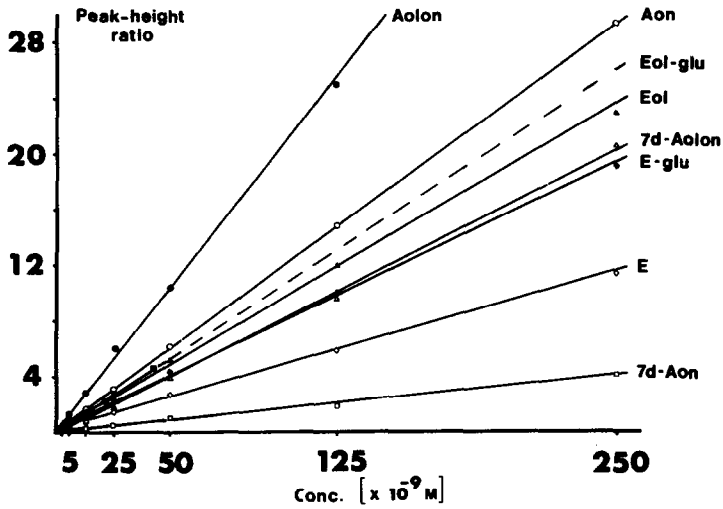

Fig. 3. Calibration lines (mean of within-day values, $n=6$) of E and metabolites in plasma, with A as internal standard.

that a daily run of unknown samples should be accompanied by standard samples. The large differences in the slopes of the standard lines between the metabolites reflect differences in recovery, fluorescence quantum yield and retention time. Therefore, the assumption of equal standard lines for all metabolites [3] is incorrect.

The within-day precision of the assay, as indicated by the coefficients of variation (C.V.), was established by the analysis of three plasma samples spiked with E and its metabolites at concentrations of $0.5 \cdot 10^{-8}$, $2.5 \cdot 10^{-8}$ and $12.5 \cdot 10^{-8}$ *M*. This procedure was repeated six times during the day. The between-day C.V. was calculated from the daily mean of duplicate analyses of plasma samples contain-

TABLE III

WITHIN-DAY AND BETWEEN-DAY COEFFICIENTS OF VARIATION OF THE SLOPES OF CALIBRATION LINES

Compound	C.V. of slope (%)	
	Within-day ($n=6$)	Between-day ($n=6$)
Eol-glu	3	18
E-glu	4	13
Aolon	2	9
Eol	3	6
7d-Aolon	5	20
Aon	4	12
E	3	8
7d-Aon	6	22

TABLE IV

WITHIN-DAY AND BETWEEN-DAY COEFFICIENTS OF VARIATION OF CONCENTRATIONS FROM CALIBRATION LINES

Compound	C.V. of concentrations (%)				
	Within-day ($n=6$)			Between-day ($n=6$)	
	$0.5 \cdot 10^{-8} M$	$2.5 \cdot 10^{-8} M$	$12.5 \cdot 10^{-8} M$	$0.5 \cdot 10^{-8} M$	$2.5 \cdot 10^{-8} M$
Eol-glu	14	6	3	–	2
E-glu	9	5	4	7	3
Aolon	8	5	3	4	2
Eol	10	5	4	4	2
7d-Aolon	8	3	2	11	5
Aon	8	5	2	5	3
E	10	5	3	10	2
7d-Aon	6	6	3	10	3

ing E and its metabolites at concentrations of $0.5 \cdot 10^{-8}$ and $2.5 \cdot 10^{-8} M$, analysed over six consecutive days. All values are shown in Table IV. As expected, the lowest C.V. values were obtained for the highest concentrations, while the between-day C.V. was smaller than the within-day C.V.

It can be concluded that our Sep-Pak extraction procedure resulted in high recoveries for all the anthracyclines studied. The HPLC procedure was rapid and could be used to separate anthracyclines with a wide range of polarities by simple isocratic elution. The detection limit for all compounds was improved considerably with respect to earlier assays, because of the high recoveries and the use of a more sensitive detector. This allows pharmacokinetic studies of epidoxorubicin during and after administrations with long infusion times.

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