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NEW METHOD FOR THE DETERMINATION OF DOXORUBICIN, 4′-EPIDOXORUBICIN AND ALL KNOWN METABOLITES IN CARDIAC TISSUE

P.A. MAESSEN*, H.M. PINEDO, K.B. MROSS and W.J.F. VAN DER VIJGH

Department of Oncology, Academic Hospital Free University, De Boelelaan 1117, 1081 HV Amsterdam (The Netherlands)

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SUMMARY

4′-Epidoxorubicin, doxorubicin (internal standard) and eight metabolites were extracted from heart tissue homogenate by a mixture of tetrahydrofuran–water (1:2, v/v) and purified by C_{18} Sep-Pak cartridges. The buffer used to prepare the homogenate contained glucaric acid-1,4-lactone and glucose, to prevent decomposition of the 4′-epidoxorubicin glucuronides. Anthracyclines were separated by high-performance liquid chromatography within 14 min and detected by fluorescence. Recoveries ranged from 49 to 75%. The detection limits of the individual anthracyclines ranged from $0.5 \cdot 10^{-11}$ to $2.5 \cdot 10^{-11}$ mol/g wet weight. The peak-height ratios of the fluorescence intensities of the anthracyclines versus doxorubicin were linear from $2.5 \cdot 10^{-11}$ to $250 \cdot 10^{-11}$ mol/g wet weight. Within- and between-day precisions of the assay varied between the anthracyclines and were in the ranges 3–12% ($n=6$) and 2–11% ($n=6$), respectively.

INTRODUCTION

Doxorubicin (A) and 4′-epidoxorubicin (E) are potent drugs against a wide spectrum of tumours. Dose-limiting cardiotoxicity appears to be lower in the case of E. Most of the discussion about the onset of cardiotoxicity is based on the pharmacokinetics of E, A and their metabolites in plasma. Recently, it was suggested that the formation of glucuronides (E-glu, Eol-glu), and their presence in plasma of patients after the administration of E, does not explain the reduced cardiotoxicity of this drug [1,2]. Perhaps the pharmacokinetics of the parent drugs and their metabolites in target organs may contribute to the understanding of differences in the relative therapeutic and toxic effects of the drugs. For that purpose, a reliable assay to determine anthracycline concentrations in tissues is required.

Methods used to determine anthracyclines in tissue are generally based on an

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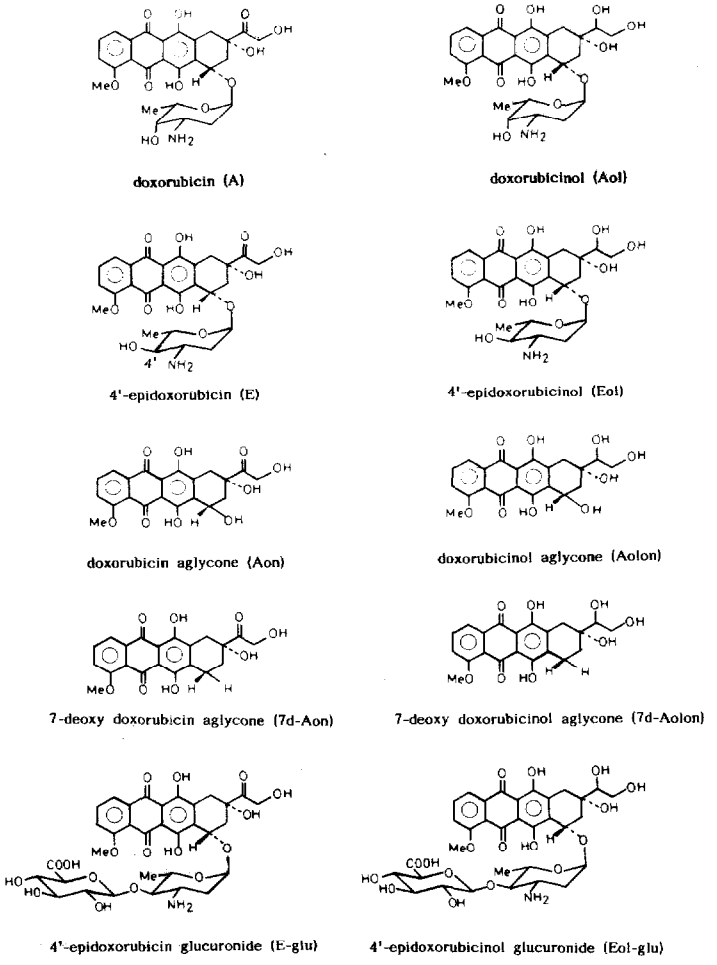

Fig. 1. Structures of E, A and their metabolites.

extraction step with organic solvents, such as chloroform–methanol [3–6], chloroform–isopropanol [7], toluene–butanol [8], ethyl acetate–butanol [9], isoamyl alcohol [10] or acetonitrile [11]. After evaporation of the solvent(s), residues are injected onto the high performance liquid chromatograpic (HPLC) column. One assay [11] includes an additional purification of the residue using Bond-Elut columns.

However, the extraction procedures mentioned above have limitations in the number of metabolites measured, the quality of HPLC separation, the accuracy (one common calibration line for all metabolites [7]) and precision (not mentioned). The purpose of the present study was to develop an extraction, purification and HPLC procedure for the determination of E, A and all their known metabolites (Fig. 1), including glucuronides, in heart tissue.

EXPERIMENTAL

Eol-glu and E-glu were isolated from urine of a patient receiving E. Isolation was performed using a preparative glass column packed with octadecyl-Si-100 polyol (Serva, Heidelberg, F.R.G.) and eluted with methanol–water (2:3, v/v) at a flow-rate of 0.5 ml/min. Purity was checked by HPLC (>98%). E, A and other metabolites were provided by Farmitalia Carlo Erba (Milan, Italy). Tetrahydrofuran (Baker, Deventer, The Netherlands), acetonitrile, D-glucose, silver nitrate and sodium dihydrogenphosphate (Merck, Amsterdam, The Netherlands) were of analytical grade. Glucaric acid-1,4-lactone was obtained from Sigma (Amsterdam, The Netherlands).

Stock solutions of E and metabolites in methanol were combined to obtain an equimolar mixture of anthracyclines. This mixture was diluted with methanol to obtain concentrations of 10^{-8}, $5 \cdot 10^{-8}$, 10^{-7}, $2.5 \cdot 10^{-7}$, $5 \cdot 10^{-7}$ and 10^{-6} M.

Hearts were obtained after decapitation of DBA-2 mice (adult males). Thirty-three hearts were homogenized by dismembration (1 min, 77 K, using a Mikrodismembrator II, Braun, Melsungen, F.R.G.) and immediately suspended in 33 ml of glucaric acid-1,4-lactone–glucose solution (3 g:0.5 g, in 1 l of distilled water). Aliquots of 4 ml of homogenate were stored at −20 °C. Samples were prepared in polypropylene tubes by evaporating the solvent from the appropriate amount of an anthracycline mixture, followed by the addition of 250 μl of homogenate (39 mg wet weight) and 50 μl of internal standard (A, $5 \cdot 10^{-7}$ *M*). In this way, anthracycline concentrations in tissue of $2.5 \cdot 10^{-11}$ to $250 \cdot 10^{-11}$ mol/g wet weight were obtained.

After incubation (30 min, vortex), 100 μl of 3 *M* silver nitrate in water were added to each tube, followed by homogenization (10 min, vortex). The anthracyclines were extracted by shaking with 1 ml of tetrahydrofuran–water (1:2, v/v) for 10 min. Phase separation was obtained by centrifugation for 5 min at 1500 *g*. The supernatant was transferred to a C_{18} Sep-Pak cartridge (pretreated with 5 ml of methanol and 5 ml of water) and washed with 2 ml of water. The anthracyclines were subsequently eluted with 4 ml of chloroform–methanol (1:3, v/v). The pellet was extracted a second time, and the combined chloroform–methanol solutions were evaporated to dryness at 55 °C. The residue was dissolved by vortexing in 100 μl of 0.02 *M* sodium dihydrogenphosphate (pH 4)–acetonitrile (9:1, v/v), of which 30 μl were injected onto the HPLC column.

E, A and their metabolites were separated and detected by the HPLC system described previously [12], consisting of a reversed-phase column (3-μm CP MicroSpher RP-18, 100 mm×4.6 mm I.D.) with guard column (5-μm LiChrosorb RP-18, 4 mm×4 mm I.D.) and fluorescence detection. Elution was under isocratic conditions with 0.02 *M* sodium dihydrogenphosphate (pH 4)–acetonitrile (2.5:1, v/v) at a flow-rate of 1 ml/min.

RESULTS AND DISCUSSION

A chromatogram of an extract of a tissue homogenate spiked with anthracyclines ($25 \cdot 10^{-11}$ mol/g wet weight) is shown in Fig. 2a. All compounds are eluted

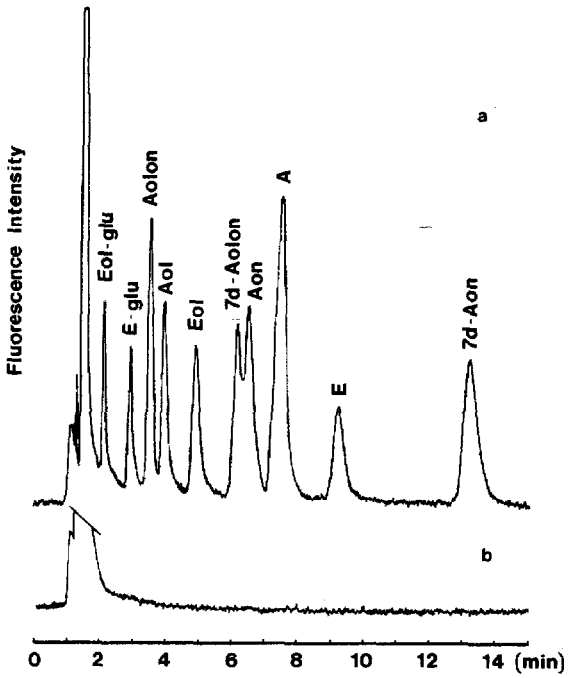

Fig. 2. HPLC profile of a tissue extract from a heart spiked with anthracyclines ($25 \cdot 10^{-11}$ mol/g; A as internal standard, $62.5 \cdot 10^{-11}$ mol/g wet weight) (a) and of a blank heart (b). Conditions: column, 3-μm CP MicroSpher RP-18, 100 mm × 4.6 mm I.D.; guard column, 5-μm LiChrosorb RP-18, 4 mm × 4 mm I.D.; mobile phase, 0.02 *M* sodium dihydrogenphosphate–acetonitrile (2.5:1); flow-rate, 1 ml/min; detector, fluorescence (excitation 480 nm, emission 580 nm). For peak identification, see Fig. 1.

within 14 min. A similarly treated blank homogenate is depicted in Fig. 2b. Obviously, no endogenous fluorescent compounds are co-eluting with any of the analysed anthracyclines.

In contrast to previously described procedures [3–11] extraction of the glucuronides from tissue was accomplished by inhibiting β-glucuronidase activity [13] with glucaric acid-1,4-lactone–glucose (3 g:0.5 g, in 1 l of distilled water). Absence of these sugars resulted in very low recoveries for the glucuronides (0–10%). The silver nitrate solution was added to release anthracyclines from DNA and proteins [10]. Extraction of the anthracyclines from heart tissue was performed using tetrahydrofuran–water (1:2, v/v). This miscible mixture is of such an intermediate polarity that it allows the extraction of anthracyclines with a wide range of polarity. When such an extract is introduced onto the C_{18} Sep-Pak cartridge, the anthracyclines are retained. In this way evaporation of the solvents is omitted. After washing (2 ml of water), the compounds are eluted using chloroform–methanol (1:3, v/v), as reported previously [12]. This method resulted in high recoveries of the anthracyclines, whereas the HPLC separation and detection of these compounds was very accurate and sensitive.

The detection limit of the assay (at a signal-to-noise ratio of 2) ranged from $0.5 \cdot 10^{-11}$ to $2.5 \cdot 10^{-11}$ mol/g wet weight (Table I), which compares favourably with other assays [3–11]. The recoveries of the anthracyclines from spiked car-

TABLE I

DETECTION LIMITS OF E, A AND METABOLITES

Compound*	Detection limit ($\times 10^{-11}$ mol/g wet weight)
Eol-glu	1.0
E-glu	1.4
Aolon	0.5
Aol	1.0
Eol	1.1
7d-Aolon	1.0
Aon	0.8
A	1.8
E	2.5
7d-Aon	1.3

*For abbreviations see Fig. 1.

diac tissue were calculated by comparing peak-heights in the chromatograms of extracted samples with those of standards with identical concentrations in methanol. Recoveries ranged from 49 to 75% (Table II). Owing to the additional tetrahydrofuran–water extraction step in the tissue-extraction procedure, recoveries are somewhat lower than those of plasma extractions using only Sep-Pak cartridges. [12].

Calibration curves, constructed by least-squares analysis of the mean values of the within-day peak-height ratios of E and metabolites to the internal standard A against the concentration in 1 g of tissue (wet weight) are shown in Fig. 3. The plots were linear for all compounds from $2.5 \cdot 10^{-11}$ to $250 \cdot 10^{-11}$ mol/g wet weight,

TABLE II

RECOVERIES OF E, A AND THEIR METABOLITES

Compound*	Recovery (mean ± C.V., $n=12$) (%)	
	$12.5 \cdot 10^{-11}$ mol/g	$125 \cdot 10^{-11}$ mol/g
Eol-glu	55 ± 10	51 ± 8
E-glu	62 ± 8	60 ± 6
Aolon	50 ± 5	49 ± 3
Aol	63 ± 5	58 ± 6
Eol	62 ± 4	62 ± 5
7d-Aolon	58 ± 8	61 ± 3
Aon	52 ± 10	57 ± 4
A	-	74 ± 6**
E	71 ± 5	75 ± 8
7d-Aon	52 ± 8	52 ± 5

*For abbreviations, see Fig. 1.
**$62.5 \cdot 10^{-11}$ mol/g wet weight.

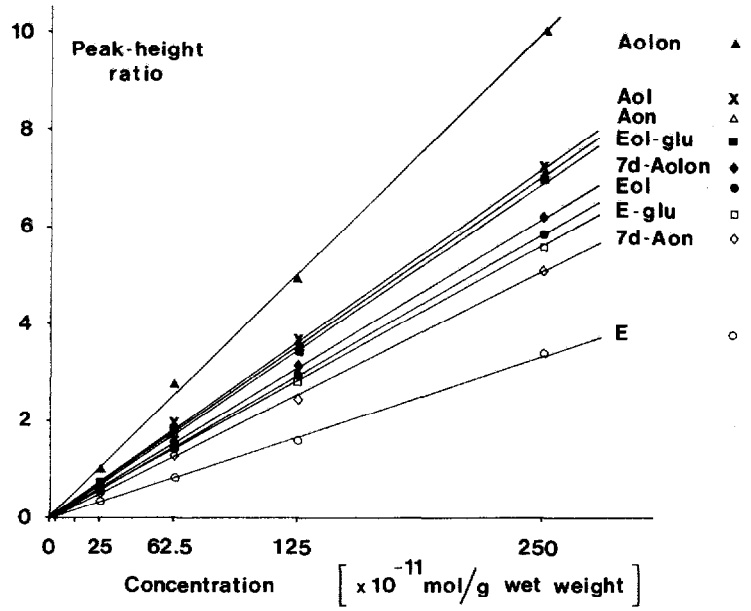

Fig. 3. Calibration lines (mean of within-day values, $n=6$) of E and metabolites, with A as internal standard ($62.5 \cdot 10^{-11}$ mol/g wet weight). For abbreviations, see Fig. 1.

with coefficients of regression better than 0.99. Differences in slopes of calibration lines between the compounds analysed reflect differences in recovery, fluorescence quantum yield and retention time. Table III shows the within-day ($n=6$) and between-day ($n=6$) variation of the slopes of the calibration lines for E and metabolites. These data show the necessity of a daily run of standard samples when analysing samples with unknown concentrations of anthracyclines.

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	5	20
E-glu	3	17
Aolon	5	13
Aol	3	12
Eol	3	9
7d-Aolon	5	10
Aon	4	6
E	3	7
7d-Aon	8	6

*For abbreviations, see Fig. 1.

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 ($n=6$)	
	$12.5 \cdot 10^{-11}$ mol/g	$125 \cdot 10^{-11}$ mol/g	$12.5 \cdot 10^{-11}$ mol/g	$125 \cdot 10^{-11}$ mol/g
Eol-glu	12	6	8	3
E-glu	8	5	11	3
Aolon	8	5	6	2
Aol	7	3	11	4
Eol	4	3	6	4
7d-Aolon	10	4	4	2
Aon	9	4	4	2
E	8	6	7	5
7d-Aon	11	5	5	3

*For abbreviations, see Fig. 1.

The within-day precision of the assay, as indicated by the C.V. was established by the analysis of tissue samples spiked with E and metabolites at concentrations of $12.5 \cdot 10^{-11}$ and $125 \cdot 10^{-11}$ mol/g wet weight. This procedure was repeated six times during the day. The between-day C.V. was calculated from the daily mean of duplicate analyses of tissue samples of the same concentrations analysed over six days. The results (Table IV) show that the lowest C.V. is obtained for the highest concentration ($125 \cdot 10^{-11}$ mol/g wet weight).

In conclusion, a rapid and elegant extraction method was developed, with comparable recoveries of E, A and their metabolites, including glucuronides, from heart tissue. In conjunction with the previously reported [12] HPLC separation and fluorescence detection assay, this method compares favourably with all other assays on tissue anthracycline levels reported to date. This assay will allow pharmacokinetic studies of parent drug and metabolites in animal and human tissues (e.g., heart and tumour) after administration of A or E, aiming at a better understanding of both therapeutic and toxic effects of the drugs.

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