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Short Communication

Simple and sensitive quantification of anthracyclines in mouse atrial tissue using high-performance liquid chromatography and fluorescence detection

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ABSTRACT

Anthracyclines are very effective against soft tissue sarcomas, with cardiotoxicity being an important side effect after repeated administration. To estimate the relative cardiotoxicity of various anthracyclines and their metabolites, we developed an isolated mouse left atrium model. To relate an effect of doxorubicin, 4′-epidoxorubicin and their four main metabolites (doxorubicinol, epidoxorubicinol and the aglycons 7-deoxydoxorubicinon and 7-deoxydoxorubicinolon) to concentrations in the tissue instead of the incubation bath, a method of quantifying the anthracyclines in small tissue samples was developed. Atria were homogenized by sonication followed by extraction of the anthracyclines with methanol. The extract was directly analyzed by high-performance liquid chromatography with fluorescence detection. Recoveries for the six compounds tested ranged from 67.5% for 4′-epidoxorubicin to 100.6% for 7-deoxydoxorubinol aglycon with coefficients of variation of 2–3% at two spiked concentrations (0.1 and 1 nmol/mg of tissue). The calibration plots were linear ($r^2 > 0.996$) over the concentration range tested (0.05–1 nmol/mg wet weight). The limits of detection (4–10 pmol/mg of tissue) were low enough to allow the determination of the anthracyclines at all relevant tissue concentrations.

INTRODUCTION

Anthracyclines, with doxorubicin (Dox) as their main representative, are often used in cancer chemotherapy. Their cumulative dose is limited by cardiotoxicity [1]. The 4′-isomer of Dox, 4′-epidoxorubicin (E), was introduced in the clinic

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because of its lower cardiotoxicity [2]. To investigate the hypothesis that metabolites may play a role in the processes leading to this toxicity, we developed an *in vitro* model using isolated mouse atria [3]. With this model we measured the effect of Dox, E and each individual metabolite (structures shown in Table I) on the contractile force of the heart muscle. To relate observed effects to tissue concentrations, a method was required for analyzing small tissue samples of about 1 mg wet weight. Common homogenization techniques such as pottering, dismembration [4,5] or the use of a blender [6] can only be applied to sample sizes exceeding 100 mg, while sonication has been used to homogenize a cell suspension [7]. Most studies utilize a liquid–liquid extraction with a water-immiscible fluid, which complicates sample work-up [6,8]. To avoid averaging out biological variations in tissue uptake, pooling of atria is not desirable. Therefore, procedures required for homogenization, extraction and quantification have to be chosen in such a way that the drug, including its metabolites formed during incubation, can be analyzed in a single mouse atrium in a simple manner. A few procedures using reversed-phase high-performance liquid chromatography (HPLC) with fluorescence detection show a baseline separation of a number of anthracyclines and a high sensitivity [4,6]. Thus, only minor modifications were expected to be necessary to analyze atrial extracts.

In this study, we applied ultrasound to homogenize a single atrium, followed by an extraction with methanol to release the anthracyclines. After acidification, an HPLC injection was performed. All compounds of interest were separated isocratically within 14 min.

EXPERIMENTAL

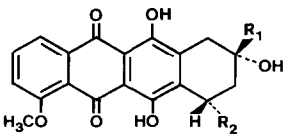
Materials

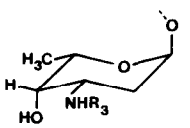
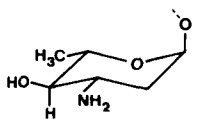
Anthracyclines, all analytically pure, were kindly provided by Farmitalia Carlo Erba (Milan, Italy). Doubly distilled water was supplied by Travenol (Utrecht, Netherlands). Acetonitrile, silver nitrate, sodium dihydrogenphosphate and phosphoric acid were obtained from Merck (Amsterdam, Netherlands), methanol from Baker (Deventer, Netherlands) and triethylamine from Pierce (Rockford, IL, USA). All reagents were of analytical grade. Phosphate-buffered saline (PBS), composition 9.0 m*M* disodium hydrogenphosphate, 3.6 m*M* sodium dihydrogenphosphate and 140 m*M* sodium chloride (pH 7.4), was supplied by the Free University Hospital Pharmacy Department (Amsterdam, Netherlands).

Stock solutions of each anthracycline were prepared in methanol at 10^{-3} *M*, and combined in order to obtain an equimolar mixture of the compounds. From this mixture further dilutions in methanol were prepared, ranging from $2.5 \cdot 10^{-7}$ to $5 \cdot 10^{-6}$ *M*. All solutions were kept in polypropylene tubes to minimize adsorption, and stored at −20°C.

TABLE I

STRUCTURES OF THE ANTHRACYCLINES



Compound	Abbreviation	R_1	R_2
Doxorubicin	Dox	$COCH_2OH$	
Doxorubicinol	Dol	$CH(OH)CH_2OH$	
4′-Epidoxorubicin	E	$COCH_2OH$	
4′-Epidoxorubicinol	Eol	$CH(OH)CH_2OH$	
7-Deoxydoxorubicin aglycon	7d-Doxon	$COCH_2OH$	H
7-Deoxydoxorubicinol aglycon	7d-Dolon	$CH(OH)CH_2OH$	H

Sample pretreatment

After incubation, each atrium was blotted dry, weighed (using a Mettler M30 microbalance, Zürich, Switzerland) and stored at −80°C in a 1.5-ml polypropylene vial (Treff, Degersheim, Switzerland). After thawing, 200 μl of PBS were added per milligram of tissue. The tissue was sonicated at half-maximal power for five cycles of 15 s with 10-s intervals, using an MSE Soniprep 150 ultrasonic disintegrator (MSE, Crawley, UK) with the sonicating tip lowered to the bottom of the vial. The vial was continuously cooled on ice. A 100-μl sample of homogenate was transferred to another 1.5-ml vial, and 400 μl of methanol were added. The anthracyclines were extracted by vortex-mixing for 15 min, followed by the addition of 150 μl of phosphoric acid (12 m*M*). Precipitated proteins were spun down at 10 000 *g* for 8 min. The vials were transferred to the autosampler (kept at 4°C) and 30 μl were injected into the HPLC system.

Calibration samples were prepared by successively pipetting 100 μl of standard mixture into a 1.5-ml vial, evaporating the methanol (50°C, nitrogen stream), adding 100 μl of blank homogenate (prepared from non-incubated mouse atria by the sonication procedure) and vortex-mixing for 15 min (in order to obtain an equilibrium between free and membrane-bound anthracycline). Thus, starting with standard solutions ranging from $2.5 \cdot 10^{-7}$ to $5 \cdot 10^{-6}$ *M*, spiked concentrations of 0.05–1.0 nmol/mg of tissue were obtained. The calibration samples were further treated as described above for the tissue samples.

Accuracy samples were prepared by evaporating the methanol from 1.0 ml of the $5 \cdot 10^{-6}$ and $5 \cdot 10^{-7}$ *M* standard solutions, respectively, and redissolving each residue in 1.0 ml of blank tissue homogenate by vortexing for 15 min (resulting in spiked concentrations of 0.1 and 1.0 nmol/mg of tissue, respectively). Aliquots of 100 μl were transferred to 1.5-ml vials and stored at −20°C.

Chromatography

The HPLC system consisted of a Applied Biosystems Spectroflow 400 pump (Separations Analytical Instruments, H. I. Ambacht, Netherlands) and a Gilson 232-401 autosampler, fitted with a 100-μl injection loop and a specially developed Eppendorf injection needle (Meyvis, Bergen op Zoom, Netherlands). A Merck-Hitachi F1000 fluorescence detector (Merck, Amsterdam, Netherlands) with the excitation wavelength (λ_{ex}) and emission wavelength (λ_{em}) set at 480 and 580 nm, respectively, was used for peak monitoring. Data processing was carried out by a Jones Chromatography JCL 6000 data system (Meyvis) in combination with an Olivetti M240 personal computer (Olivetti, Rotterdam, Netherlands). A Microspher C_{18} analytical column (two 10 cm × 4.6 mm I.D. columns in series, 3 μm, Chrompack, Middelburg, Netherlands) was used together with a 10 mm × 2.0 mm I.D. Chromsep reversed-phase guard column (40 μm) (Chrompack). The mobile phase, 2 m*M* triethylamine in 15 m*M* sodium dihydrogenphosphate (pH 4)–acetonitrile (2:1, v/v), was passed over a 0.45-μm filter before use and pumped through at a flow-rate of 1.0 ml/min.

RESULTS AND DISCUSSION

Sample pretreatment

Using ultrasonic disintegration, we were able to homogenize atria immersed in PBS (pH 7.4). Methanol was subsequently added to extract the anthracyclines. Attempts to homogenize an atrium directly in methanol were not successful, as the tissue remained intact.

Recoveries of the anthracyclines, determined with respect to a direct injection of the stock solution, ranged from 67.5% for E to 100.6% for 7d-Dolon (Table II). The recovery values show a clear difference between both Dox and E, being less than 80%, and the almost quantitative recovery of the four metabolites tested. This is probably caused by a higher degree of tissue binding of the former compounds and is supposedly related to their higher lipophilicity. We also observed a slight increase in recovery percentages for Dox and E at higher concentrations, probably due to a small absolute amount that cannot be released from binding to tissue macromolecules such as DNA.

Since significant amounts of energy are released in the solution using the disintegrator, an undesirable degradation of analytes might occur. However, sonication had no effect on the recovery of anthracyclines dissolved in PBS, nor did we observe degradation products. Adsorption of anthracyclines to the metal sonicat-

TABLE II

ANALYTICAL DATA OF ANTHRACYCLINES ADDED TO TISSUE HOMOGENATES

Compound	Recovery (mean ± S.D.) (%)		Calibration line[a]		Detection limit[b] (pmol/mg)
	0.1 nmol/mg (n = 6)	1 nmol/mg (n = 6)	r^2	C.V. of slope (%)	
Dox	74.9 ± 2.5	79.1 ± 1.7	0.997	5.5	8
Dol	99.5 ± 2.3	96.5 ± 2.6	0.998	3.7	4
E	67.5 ± 3.1	74.1 ± 2.0	0.996	5.7	10
Eol	98.3 ± 2.7	97.6 ± 2.3	0.998	3.6	5
7d-Doxon	99.4 ± 3.1	98.7 ± 2.2	0.998	10.9	7
7d-Dolon	100.6 ± 8.0	99.7 ± 2.3	0.996	7.0	6

[a] Seven concentrations in the range of 0.05–1 nmol/mg of tissue (mean of six days).

[b] Determined at a signal-to-noise ratio of 3.

ing tip might also lead to erroneous results [9], either by a decreased recovery or by cross-contamination. Immersing the tip in 200 μl of a 0.5 μM solution of anthracyclines in PBS decreased the concentration by almost 50%. However, when a tissue homogenate instead of PBS was used, no loss was observed. To exclude any memory effect the sonicating tip was cleaned with acidic acetone (2% phosphoric acid) between samples [10].

After extraction with four volumes of methanol, the mixture had to be acidified before HPLC injection in order to improve chromatographic behavior. This was achieved by adding phosphoric acid (150 μl, 12 mM), to obtain an apparent pH of about 4. Sample pH values >4 resulted in dramatically reduced peak heights (without affecting peak shapes). No clear explanation for this phenomenon is available; since the eluent also had an apparent pH of approximately 5, no incompatibility is expected.

Chromatography

Representative chromatograms of an extract of mouse atria with and without an equimolar mixture of all relvant anthracyclines (0.15 nmol/mg of wet tissue) are depicted in Fig. 1. The mixture constituents are well separated and no interfering peaks from the matrix are observed. Fig. 2 shows the chromatogram of an extract prepared from a mouse atrium incubated with 30 μM Dox for 3 h. It is clear from the amplified chromatogram that Dox is only metabolized to a very limited extent (< 0.5%) during *in vitro* incubation, thus justifying our pharmacological model with regard to unambiguous measurement of a single anthracycline.

Triethylamine was added to the mobile phase to improve peak shape and to control retention behavior. As can be seen from Table I, part of the compounds contain an amino group and thus show an important silanol interaction, resulting

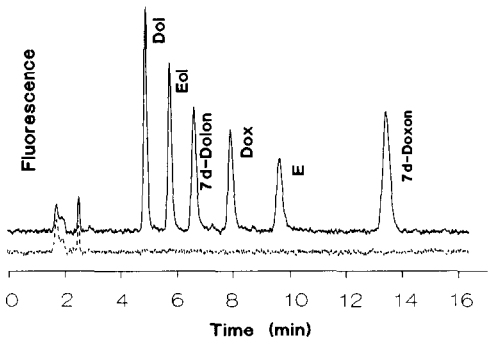


Fig. 1. Chromatograms of extracts from mouse atrial tissue (30 μl, 231 μg of tissue), spiked with 0.15 nmol/mg of tissue of each anthracycline (—) or blank (---). Chromatographic conditions: 3-μm Microspher C_{18} analytical column (20 cm × 4.6 mm I.D.), Chromsep reversed-phase guard column (10 mm × 2.0 mm I.D.), mobile phase 2 m*M* triethylamine in 15 m*M* sodium dihydrogenphosphate (pH 4)–acetonitrile (2:1, v/v), flow-rate 1.0 ml/min, fluorescence detection with λ_{ex} = 480 nm and λ_{em} = 580 nm. For peak identification, see Table I.

in longer retention and slightly tailing peaks. Although the silanol effect was not fully suppressed by 2 m*M* triethylamine, retention times remained reproducible over months. An increase in the phosphate concentration had a similar effect, probably because ion pairs are formed, which are less prone to silanol interaction. Neither parameter affected the retention behavior of the 7-deoxy compounds, thus offering the possibility of "tuning" the separation. The result is depicted in Fig. 1, which shows chromatograms obtained after injecting both a spiked and a blank tissue extract. The absence of interferences demonstrates the clean-up efficiency.

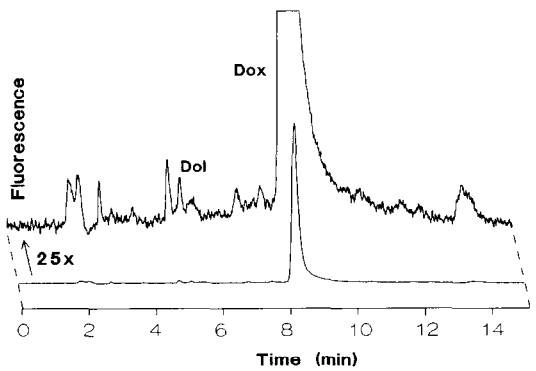


Fig. 2. Chromatogram of an extract of mouse left atrium (30 μl, 231 μg of tissue) after 3 h of incubation with 30 μ*M* Dox (plotted at two sensitivity levels). For chromatographic conditions, see Fig. 1. Peak identification: Dox = doxorubicin; Dol = doxorubicinol.

Validation

The described procedure was validated by the duplicate analysis of tissue homogenates (prepared from blank mouse atria) spiked with an anthracycline mixture at two concentration levels (0.1 and 1 nmol/mg of wet tissue) on six days. The method proved to be linear over the concentration range tested (0.05–1 nmol/mg wet weight). Calibration curves for each metabolite could be fitted with the linear least-squares method ($r^2 \geq 0.996$, Table II) with good day-to-day repeatability, as illustrated by the coefficients of variation in the slopes. For Dox and E, a slight deviation from linearity was observed, reflected in the r^2 values being the lowest out of the series. This could be attributed to a small increase of the recovery with the concentration (Table II). Therefore, depending on the individual peak heights, unknown concentrations of these compounds were determined using either the four lowest ($2.5–10 \cdot 10^{-7}$ *M*) or the four highest points of the calibration curve (the slope of the latter being approximately 10% higher). Also shown in Table II is the within-day repeatability value, expressed as the S.D. ($n=6$) of the recovery. The values ranged from 1.7 to 8% and were independent of the percentage recovery. The detection limits were in the range of 4–10 pmol/mg of tissue (wet weight), which is low enough to allow quantification of anthracyclines in tissues at all concentrations relevant for the incubation experiments. These detection limits are about 100-fold lower than those obtained for daunorubicin in tissue in an earlier study [5]. In that paper, however, an off-line preconcentration was performed with Sep-Pak cartridges. Since our chromatograms of blank extracts are free from interfering peaks and hardly show a peak at t_0, detection limits are expected to be further improved by concentrating the methanolic sample in an additional evaporation–redissolution step. Additionally, the injection volume can be increased.

Summarized in Table III are the results of between-day repeatability and accu-

TABLE III

BETWEEN-DAY ACCURACY FOR ALL COMPOUNDS

Values were obtained by interpolation in the calibration line.

Compound	Percentage of spiked concentration (mean ± S.D., $n=6$)	
	0.1 nmol/mg	1 nmol/mg
Dox	93.3 ± 1.1[a]	94.5 ± 4.5
Dol	94.2 ± 3.0	93.2 ± 3.3
E	92.9 ± 2.0[a]	93.9 ± 4.2
Eol	92.2 ± 3.0	93.2 ± 3.5
7d-Doxon	90.5 ± 3.2	90.1 ± 2.8
7d-Dolon	87.0 ± 6.4	93.1 ± 2.6

[a] After deletion of one outlying value (outside mean ± 2 S.D.).

racy of the assay, as determined by interpolating peak heights of the spiked samples on the calibration line for each individual metabolite. One value, being lower than the mean minus twice the S.D., was deleted both for Dox and E (78.1 and 73.1%, respectively). The resulting between-day variation was very low, ranging from 1.1% for Dox to 6.4% for 7d-Dolon. The deviations from the spiked concentrations ranged from 5 to 10%, with one value higher than 10% for 7d-Dolon. This deviation may be explained by the work-up procedure which, in contrast to the one used for the calibration samples, did not include an evaporation step at 50°C. Indeed, pilot experiment showed that recoveries were slightly increased when the accuracy samples had been heated at 50°C for half an hour. This effect might be caused by aggregation of the anthracycline molecules at low temperatures, which was reversed upon heating.

CONCLUSIONS

Using a simple pretreatment, we were able to analyze small tissue samples for anthracyclines in a highly reproducible and accurate manner, with nearly quantitative recoveries for most compounds. All analytes were baseline-resolved, without any interferences.

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