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Note

Analysis of adriamycin and adriamycinol in micro volumes of rat plasma

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Despite ten years of intensive investigation into the therapeutic effects and side-effects of the anthracycline derivative adriamycin, cardiac toxicity is still one of its most severe toxic effects. This limits the dose and consequently its application in cancer chemotherapy. As part of an investigation of the cardiac toxicity caused by adriamycin in tumour-bearing rats, pharmacokinetic data were needed to find out whether there is a correlation between histological changes of heart tissue, the dose of adriamycin and the method of administration. Hence, an assay for adriamycin and its active metabolite adriamycinol was needed to establish pharmacokinetic parameters, such as peak levels, rate of distribution and elimination. If several rats are used for one test, the results due to interindividual differences will not be reliable. Therefore, we decided to establish pharmacokinetic parameters for one single rat.

From the literature [1, 2] one can conclude that after the initial distribution phase, a biphasic disappearance pattern is to be expected. Therefore, 10—15 plasma samples are needed to obtain a complete plasma concentration disappearance curve. This limits the volume of the plasma samples to be analyzed to a maximum of 100 μl (the total blood volume of a rat is 4—6 ml).

Recently described high-performance liquid chromatographic methods that involve fluorescence detection for selective determination of adriamycin

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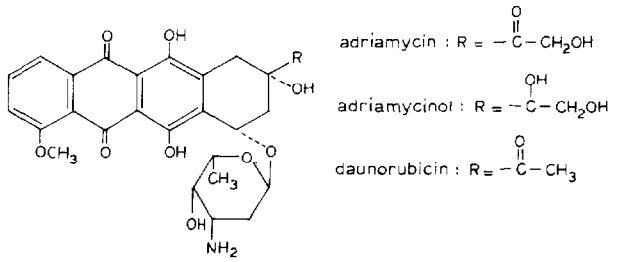

and adriamycinol, are preferred to non-selective methods like total fluorescence analysis [3, 4] and radio-immunoassays [4, 5].

Reversed-phase [6—11] as well as straight-phase [12—14] methods can be used. With straight-phase liquid chromatography and gradient elution, one can analyse nearly all the metabolites, including the inactive aglycones, glucuronated and sulphonated metabolites. Reversed-phase liquid chromatography can easily be applied for the selective assay of adriamycin and its active metabolite adriamycinol. If these methods are to be applied successfully in pharmacological practice, further requirements are easy sample treatment and simple apparatus; gradient elution techniques, for instance, should be omitted if possible.

In this paper, a simple and selective high-performance liquid chromatographic method involving fluorescence detection is described. Rat plasma samples each with a volume of 100 μl were used, plasma levels being within the therapeutic range.

To test the applicability of this method in pharmacological practice, plasma disappearance curves and preliminary pharmacokinetic data for several rats have been determined. A typical plasma concentration—time curve and related pharmacokinetic data are presented.

EXPERIMENTAL

Chemicals and apparatus

Adriamycin·HCl was obtained from Farmitalia in vials of 10 mg with 50 mg lactose. Farmitalia kindly gave us samples of adriamycinol. Daunorubicin·HCl was obtained from Specia in vials of 20 mg with 100 mg D-mannitol. Chromatographic solvents and other chemicals were of analytical grade and were used without further purification. The chromatographic system consisted of a Model 6000A solvent delivery system and a U6K septumless injection system (both from Waters Assoc., Milford, MA, U.S.A.), and a Perkin-Elmer Model 204 fluorescence detector supplied with a Hellma 25-μl flow cell (Hellma, type 176.70). A Merck LiChrosorb RP-8 (50432 C8) reversed-phase column (12.5 × 4 mm I.D., particle size 5 μm) was used. The mobile phase consisted of acetonitrile—distilled water—0.1 *M* H_3PO_4 (31:61:8) containing 10 μg desipramine · HCl per ml (pH 2.3), and was filtered through a 0.2-μm filter and deaerated ultrasonically before use. The flow-rate was 1.5—2 ml min^{-1}. Fluorimetric detection was performed at excitation wavelength 470 nm and emission wavelength 565 nm. Quantitation was based on peak height ratios using the structural analogue daunorubicin as an internal

standard. The chromatographic analyses were performed at ambient temperature.

Sample pre-treatment of micro-volumes of rat plasma

The rats received i.v. injection of adriamycin. By cannulating the vena jugularis, heparinized blood samples of about 200 μl were collected in polypropylene tubes over a period of 3 days. The samples were centrifuged immediately and stored at −20°C prior to analysis. For the extraction of adriamycin and adriamycinol, 100 μl plasma was mixed with 100 μl borate buffer pH 9.0 in a conical polypropylene tube of 1.5 ml. The buffer was composed of 24.7 g boric acid, 6.8 g sodium hydroxide, 29.7 g potassium chloride and 10 mg desipramine·HCl per litre [7, 15]. Appropriate amounts of the internal standard daunorubicin were added in 10-μl volumes of an aqueous solution. A 0.50-ml volume of a chloroform—1-heptanol mixture (1:1) was added. The mixture was vortexed for 45 sec and centrifuged for 5 min at 2500 *g*. The aqueous upper layer was removed and the organic layer was transferred to a new conical polypropylene tube of 1.5 ml containing 100 μl 0.2 *M* phosphoric acid. After 1 min vortexing and 5 min centrifugation at 2500 *g*, 10 to 90 μl of the aqueous phase, depending on the expected concentration level, was injected into the chromatographic system. All the glassware used was silanized before use by treating it with a solution of 2% trimethylchlorosilane in toluene, followed by a washing procedure with methanol.

Blood sampling

Male Wistar rats all weighing about 250 to 300 g were cannulated in the left vena jugularis. The cannule was flushed with a heparin solution twice a day and after each blood sampling. Adriamycin (2 mg kg^{-1}) was administered by i.v. bolus injection. The valve of the cannule was opened and about 200 μl blood was collected in heparinized polypropylene tubes. If the rats damaged the cannule, blood was collected by orbital puncture.

Calculation

The plasma levels of adriamycin were analysed using the HP 9810, programmed with the Wagner stripping method [16].

RESULTS

The straight-phase liquid chromatographic method for the analysis of adriamycin and adriamycinol, described by Baurain et al. [14], is the only one that uses 100 μl of plasma. However, the authors showed its applicability in the μg range only. All other methods mentioned earlier in this paper — reversed-phase as well as straight phase — need 1—4 ml of plasma.

To determine adriamycin and adriamycinol in the ng range, with acceptable accuracy, using 100 μl of plasma, special techniques have to be developed to prevent loss of adriamycin and adriamycinol, because of their strong adsorptive properties. The flexibility of the sample pre-treatment is limited by the adsorptive properties. Polypropylene tubes should be used if possible; if glass-

ware is used it should all be silanized [17]. However, even with silanized or siliconized glassware, adriamycin may still be adsorbed from an aqueous solution. Desipramine·HCL, with comparable adsorptive properties, was added to the buffer in the extraction procedure and to the mobile phase in the chromatographic procedure, in order to decrease the number of active, adsorptive sites. The syringe was pre-treated with trimethylchlorosilane for the same reason and had to be washed with a 4 *M* hydrochloric acid—methanol mixture (1:9). The syringe was washed many times with water between each injection, to prevent memory effects appearing on this trace level analysis. The optimum pH value of the buffer mixed with plasma was found to be pH 9.0. Eksborg [18] mentioned pH 8.6 as an optimum, but he used an organic extraction solvent with a different composition.

As in several other investigations [9, 11, 13] the structural analogue daunorubicin was used as internal standard for comparable lipophilicity, and for comparable chemical and fluorescence properties. We found chloroform—1-heptanol (1:1) to be the best possible composition for the extraction of adriamycin, adriamycinol and daunorubicin [15]. Using this organic solvent in a phase-volume ratio of 1:5, we achieved a 95% recovery of adriamycin for the whole clean-up procedure.

Eksborg [6, 17] determined the influence of the pH and the composition of the extraction mixture. His results indicate that an adriamycinol recovery of about 90% can be expected under these circumstances; this was confirmed in practice.

It is important to vortex the mixture of plasma and buffer for 45 sec since longer vibration yields in a smaller usable organic layer, because of a kind of emulsion of precipitated protein which is formed in the organic solvent. In order to achieve an almost quantitative transfer of adriamycin and adriamycinol to a small volume of the aqueous phase, the aqueous layer must be kept at an acidic pH.

By using 0.2 *M* H_3PO_4 as the aqueous phase, we obtained quantitative recovery and negligible aglycone formation. When aqueous, acidic solutions are stored for longer than about 3 h before analysis, it is preferable to use 0.1 *M* H_3PO_4, despite its lower yield. Also the life-time of the column will increase if the pH is increased.

Eksborg compared several chemically bonded phases for optimum separation of the drug, metabolite and plasma peaks and recommended the use of RP-8 bonded phases.

We investigated several commercially available RP-8 columns and selected the RP-8 column from Merck because of its very small plate height. The composition of the mobile phase (acetonitrile—water—0.1 *M* H_3PO_4, 37:60:3) was optimized for this column. For other types of columns the composition had to be changed for maximum separation. To prevent aglycone formation during the chromatographic process, the concentration of phosphoric acid should never exceed 0.1 *M*. The capacity factors (k') of adriamycin, adriamycinol and daunorubicin proved to be 1.7, 0.7 and 5.7, respectively, under these circumstances.

As in the sample treatment, the strong adsorptive properties of adriamycin interfere with the injection system and disturb the chromatographic pro-

cesses and detection. Addition of desipramine·HCl diminishes this interference to a large extent.

In this procedure no gradient elution is needed; for routine analysis the flow-rate was kept at 2 ml min^{-1} to obtain short analysis times. Fig. 1 shows a chromatogram of the analysis of a rat plasma sample. Calibration curves for standard solutions of adriamycin in aqueous solution were linear over the studied concentration range from 0.1 ng/100 μl to 10 μg/100 μl and passed through the origin (r^2 = 0.9999). Standard deviations in replication measurements were 1.5% in the higher range, and 3% in the lower range.

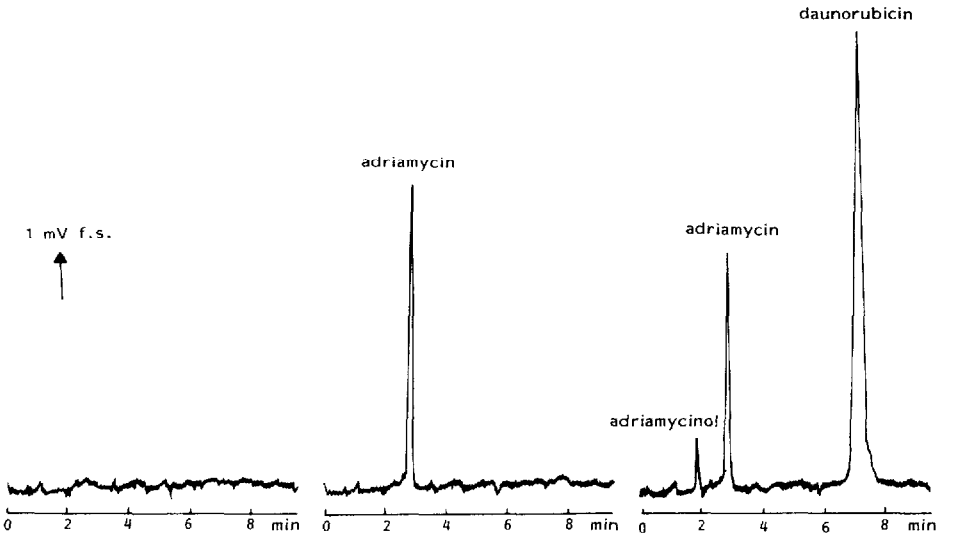

Fig. 1. A chromatogram of the analysis of rat plasma. Left, blank rat plasma; middle, blank rat plasma, spiked with 40 ng adriamycin/100 μl; right, plasma of treated rat, spiked with 60 ng daunorubicin/100 μl.

Calibration curves for the analysis of spiked plasma samples were linear over the studied concentration range of 1 ng/100 μl plasma up to 100 μg/100 μl plasma and passed through the origin (r^2 = 0.9999). Standard deviations in the analysis of plasma samples were 3% in the higher range, and 8% in the lower range.

PRELIMINARY PHARMACOKINETIC RESULTS IN RATS

We have developed the described assay of adriamycin in rat plasma in order to investigate the relation between the dosage, the method of administration of adriamycin and the toxic effects on heart tissue. To investigate the reliability of the method in pharmacological practice, we established the plasma concentration—time curves for several rats, on the basis of very small volumes of rat plasma. The blood samples were collected according to the procedure described in the experimental chapter, namely by cannulating the vena jugularis. If the rats "disconnected" the cannule, blood samples were collected by orbital puncture.

A typical plasma concentration—time curve for adriamycin and adriamycinol is shown in Fig. 2. The line, shown in this figure, is the best fit, as calculated by the computer program. The pharmacokinetic data, calculated using this computer program, are collected in Table I. A three-compartment model gave the best fit. This highly sensitive method makes it possible to measure 6—8 half-lives. The pharmacokinetic data found in this way give new insight into the distribution and elimination of the drug. The distribution volume (V_γ), the half-life time of the elimination phase ($t_{½\gamma}$) and the total body clearance (Cl_{tot}) are remarkably large, compared to earlier published data [19, 20]. Further pharmacological experiments are in progress.

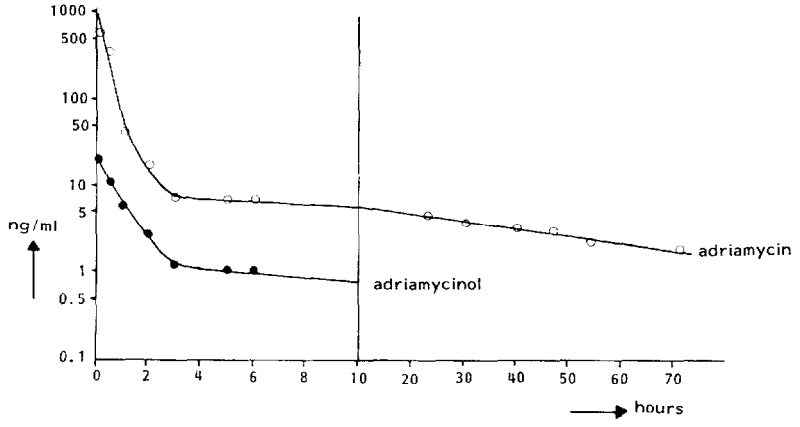

Fig. 2. A typical log plasma concentration—time curve for one rat (adriamycinol could only be analysed for up to 10 h).

TABLE I

TYPICAL PHARMACOKINETIC DATA FOR THE ANALYSIS OF ADRIAMYCIN OF THE log PLASMA CONCENTRATION—TIME CURVE FROM FIG. 2

Wistar rat; 267 g, 2 mg adriamycin/kg by i.v. bolus injection

$C = A \cdot e^{-\alpha t} + B \cdot e^{-\beta t} + P \cdot e^{-\gamma t}$			
A:	996.6 ng ml^{-1}	α: 5.17 h^{-1}	$t_{½\gamma}$ = 34.3 h
B:	99.6 ng ml^{-1}	β: 1.12 h^{-1}	V_γ = 149 l kg^{-1}
P:	7.8 ng ml^{-1}	γ: 0.020 h^{-1}	Cl_{tot} = 0.050 l min^{-1} kg^{-1}

It can be concluded that the method described in this paper is reliable, sensitive and easy to use in pharmacological practice and may give us further insight into the pharmacology of adriamycin and its active metabolite in the rat and other species.

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