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High-performance liquid chromatographic determination of doxorubicin in tissues after solid phase extraction*

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Abstract: A method is described for the determination of doxorubicin in tissues. The drug was selectively extracted from the biological matrix by solid phase extraction using 1-ml octadecyl silane extraction columns. Prior to extraction, tissue samples were digested by an enzymic digestion procedure. Daunorubicin was used as an internal standard. Quantitation was by high-performance liquid chromatography (HPLC) using ion-pair chromatography on a reversed-phase column. Detection was by fluorescence.

Recovery of doxorubicin from tissue was 81.7 ± 2.1% mean ±SD. Doxorubicin concentrations as low as 0.01 mg kg^{-1} could be determined. A typical value of the relative standard error of measurement was 3.7% at 2.1 mg kg^{-1}.

Keywords: *Doxorubicin, determination in tissues, solid phase extraction, enzymic tissue digestion, ion-pair chromatography.*

Introduction

Doxorubicin (adriamycin) is a very effective anthracycline antitumour agent with a wide spectrum of activity. Its therapeutic use, however, is limited by the severe cardiomyopathy which accompanies long-term treatment. In rat studies it was demonstrated that this severe cardiotoxicity could be reduced with preservation of the antitumour effect by modification of the pharmacokinetic and distribution profile of doxorubicin by encapsulation of the drug into liposomes [1]. In order to assess the most effective type of liposome and to establish an optimum treatment protocol, the pharmacokinetics and tissue distribution of doxorubicin in the rat in relation to its pharmacological and toxicological activity have been studied in our Institute after different treatment regimens.

Recently, a method was reported for the quantitative determination of doxorubicin in plasma [2] which had proved to be very suitable for application to extensive pharmacokinetic animal studies, in which large series of samples are involved. In this method, doxorubicin was quantitatively extracted from plasma by using a newly

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developed fast, specific and sensitive solid phase extraction procedure. Quantitation was performed by HPLC with fluorescence detection using ion-pair chromatography on a reversed-phase column. The present procedure for tissues was developed from this method. Since doxorubicin is a drug susceptible to degradation under various conditions of pH, temperature and the presence of oxidative agents, a method was selected for tissue sample pretreatment using enzymic digestion [3], with mild, physiological conditions.

Materials and Methods

Apparatus

The HPLC system consisted of solvent delivery (Kratos SF 400), sample processing (Waters WISP 710B) with a column thermostat (Spark SpH 99) and fluorescence detection (Kratos SF 980) with a Xenon light source (Kratos FSA 190) operating at an excitation wavelength of 480 nm. The fluorescence above 500 nm was recorded. The detector signal was recorded and processed by software (Chromatochart, Interactive Microware) on a microcomputer (Apple IIe). The column was 150 mm long, 4.6 mm i.d., slurry-packed with 5 μm Hypersil ODS (Shandon). Column temperature was maintained at 50°C. The mobile phase (flow rate 1.0 ml min^{-1}) was a mixture of 0.021 mol l^{-1} phosphate buffer (pH 2.6; 2.24 g of disodium hydrogenphosphate dodecahydrate per litre of 0.015 mol l^{-1} orthophosphoric acid) and methanol (25/75 v/v). Sodium 1-heptanesulfonate was added to the eluent in a concentration of 0.004 mol l^{-1} resulting in a final eluent with pH 4.2.

The solid phase extractions were performed using 1-ml reversed-phase octadecyl silane extraction columns (J. T. Baker) fitted to a Baker-10 SPE extraction manifold.

Reagents and reference solutions

Reagents were of analytical grade (E. Merck). Drugs were doxorubicin hydrochloride (Roger Bellon) daunorubicin (Specia) and ephedrine hydrochloride (OPG). Enzymes were collagenase (type 1; Clostridiopeptidase A) and protease (type VIII; subtilisin Carlsberg) both from Sigma. Antibiotics were sodium penicillin G, streptomycin sulphate (both from Gist-Brocades) and fungizone (Gibco).

A working solution of doxorubicin (5.0 mg l^{-1}) was prepared in methanol and of the internal standard daunorubicin (45 mg l^{-1}) in methanol:water (1/1 v/v). These solutions were freshly prepared monthly. Aliquots of the working solutions were used to prepare calibration standards.

An enzyme suspension was freshly prepared daily by suspending collagenase (3 mg ml^{-1}) and protease (2 mg ml^{-1}) in distilled water.

An antibiotic solution was prepared by dissolving 5,000,000 E of penicillin, 5 g of streptomycin and 13 mg of fungizone in 750 ml of water.

An ascorbate buffer solution (pH = 4.5) was freshly prepared daily by mixing 1.0 ml of a fresh 1 mol l^{-1} ascorbic acid solution with 0.5 ml of 1 mol l^{-1} sodium hydroxide.

To prevent adsorption of doxorubicin and daunorubicin on glassware polypropylene labware was used for the storage and handling of standards and samples exclusively.

Tissue pretreatment

For an expected doxorubicin concentration range of 0.01–8 mg kg^{-1}, 0.5 g of solid tissue was transferred into a preweighed 26 ml polyethylene stoppered polystyrene test

tube. The sample was weighed and 2.0 ml of enzyme suspension, 40 μl of antibiotics solution, 50 μl of ascorbate buffer and 10 μl of ephedrine solution (500 mg l^{-1} methanol) were added. The tube was closed and incubated at 37°C for 15 h (overnight) until the sample was completely digested. After incubation, the digest was shaken on a reciprocal shaker at 200 rpm for 10 min. Daunorubicin solution (50 μl) and methanol (7.5 ml) were then added. The mixture was shaken for 10 min on a reciprocal shaker at 200 rpm and centrifuged at 4000 rpm for 10 min. An aliquot of 1.5 ml of the supernatant fluid was transferred into a 2.2 ml polypropylene reaction vial. The methanol content of the sample was evaporated under a gentle stream of nitrogen at 70°C to give a final sample volume of about 700 μl. Processing was then as described under solid phase extraction.

For tissue samples with expected doxorubicin concentrations higher than 8 mg kg^{-1}, appropriate predilution of the tissue digest with distilled water was achieved before the described methanol pretreatment.

Solid phase extraction

An extraction column was fitted to the Baker-10 SPE extraction manifold. Preceding the extraction, the column was pretreated consecutively, twice with 1 ml of methanol, 1 ml of methanol:water (1/1 v/v) and 1 ml ephedrine solution (5 mg l^{-1} in methanol: water 1/1 v/v). To ensure a high and reproducible recovery, all fluids should pass slowly through the column and intrusion of air, for example by vacuum suction, should be strictly prevented.

The pretreated sample was applied to the top of the column and the eluate discarded. Water- and methanol-soluble components from the biological matrix were removed by washing the column with consecutively 1 ml of water, four times 1 ml of methanol:water (1/1 v/v) and 100 μl of HPLC eluent. The drugs were eluted with 1200 μl of HPLC eluent into a 2.2 ml polypropylene reaction vial (Eppendorf) placed under the column. The methanol content in the eluate was evaporated under a gentle stream of nitrogen at 70°C to give a final sample volume of about 300 μl.

The extract was vortex mixed for 10 s and an aliquot of 200 μl transferred into a 0.4 ml polypropylene micro insert (reaction vial for Beckman Ultra Micro System with the cap and the upper 6 mm cut off) for the WISP sample vial. An aliquot of 75 μl was taken for HPLC analysis.

Results

Figure 1 shows chromatograms of extracts of liver from an untreated rat and from a rat treated with doxorubicin. Good baseline separation was achieved between doxorubicin, the internal standard daunorubicin and the peak at 2.69. By comparing the chromatograms with those of plasma extracts from patients on doxorubicin treatment, the peak at 2.69 min is probably due to doxorubicinol, a pharmacologically active metabolite of doxorubicin.

Figure 2 shows the elution profiles of doxorubicin and daunorubicin from the octadecyl silane extraction column, collecting consecutive fractions of 200 μl and measuring the drug in each fraction. Collection of the elution fractions was started directly after washing of the column with water:methanol (1/1 v/v). As for plasma [2], doxorubicin eluted mainly in the second and third fractions and daunorubicin mainly in the third and fourth fractions. In the first fraction neither drug was eluted. Based on

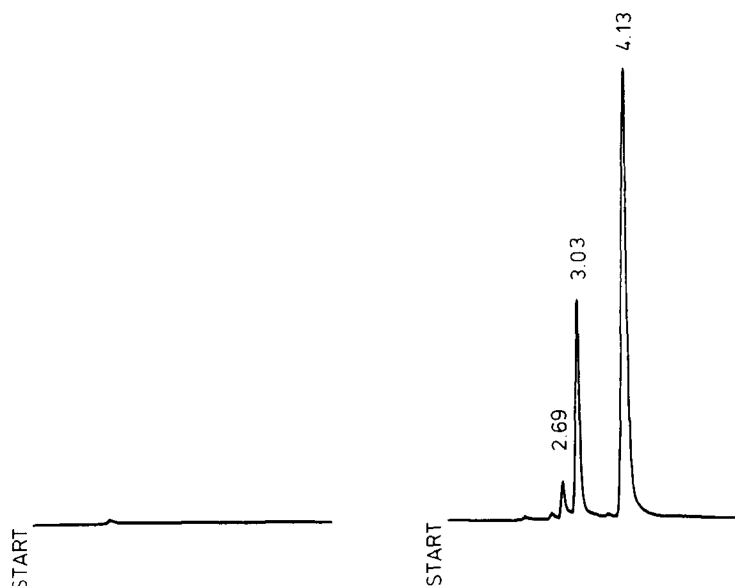

Figure 1
Chromatogram of extracts of 0.5 g of liver from an untreated rat (left) and of 0.5 g of liver from a rat treated with doxorubicin (right). Doxorubicin (2.5 mg kg^{-1}): 3.03 min; internal standard daunorubicin (4.5 mg kg^{-1}): 4.13 min.

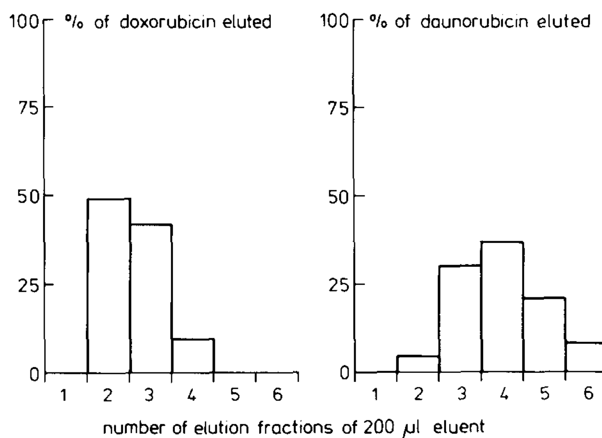

Figure 2
Elution profiles of doxorubicin and daunorubicin from the octadecyl silane solid phase extraction column after extraction of a tissue digest. The concentration of the drugs was determined in consecutive elution fractions of 200 µl eluent.

these profiles, the first 100 µl was used as a wash and elution was subsequently performed with 1200 µl of eluent.

The recovery of doxorubicin from tissue was determined from samples (N = 10) of liver from an untreated rat to which doxorubicin was added to a concentration of 2.9 mg kg^{-1}. The internal standard daunorubicin was added to the final extract at the end of the extraction procedure. The samples were carried through the digestion and extraction

procedures and the chromatogram peak area ratios of doxorubicin and daunorubicin were compared with the peak area ratios obtained on direct injection of doxorubicin reference solutions of corresponding concentration. The extraction recovery of doxorubicin from tissue was 81.7 ± 2.1% (mean ±SD).

Calibration graphs were constructed using six calibration standards prepared from liver from untreated rats and covering a concentration range of 0.01–8 mg kg^{-1} of liver. These standards were carried through the entire digestion and extraction procedure. A typical value for the relative standard error was 3.7% at 2.1 mg kg^{-1} ($y = 20.5x - 2.2$; correlation coefficient = 0.9996).

All relevant doxorubicin concentrations could be determined satisfactorily down to a concentration of 0.01 mg kg^{-1}.

Discussion

HPLC using ion-pair chromatography on a reversed-phase column has proved to be the technique of choice for the determination of doxorubicin in plasma [2]. By using 1-heptanesulfonate as an ion-pairing agent at an eluent pH of 4.2 an optimal separation between doxorubicin and daunorubicin in the absence of any peak tailing could be obtained. Doxorubicin and daunorubicin could be expected to be stable during storage of the extracted sample and during the chromatographic separation. Chromatographic conditions as assessed for the analysis of plasma samples appeared to be optimal for the analysis of tissue samples.

For the extraction of doxorubicin from plasma a fast, specific and sensitive solid phase extraction procedure using an octadecyl silane sorbent has been newly developed [2]. It was found that extraction recoveries of both doxorubicin and the internal standard daunorubicin were only 20–40% if the procedures were carried out at pH values between 8 and 9. Based on a reported optimum extraction pH of 8.4 [4] liquid–liquid extraction procedures are generally carried out at pH 8–9. Low extraction recoveries at these alkaline conditions could be explained by instability of the drugs at pH values higher than 4 [5–7]. In the proposed solid phase extraction procedure, sorbent pretreatment, analyte retention and column wash were done under physiological conditions without the use of any buffer solutions. The elution of the drugs was with a pH 4.2 eluent in which both doxorubicin and daunorubicin could be expected to be stable.

Degradation of doxorubicin appeared to be the major problem in the development of the tissue digestion procedure. Even enzymic digestion of the tissues under mild, physiological conditions [3] caused serious degradation. This could be related to an increase of the pH during digestion and/or to the long digestion time (15 h) during which doxorubicin was susceptible to hydrolytic and oxidative degradation. Addition of an ascorbate solution to the tissue prior to digestion prevented the increase of the pH during digestion by acting as a buffer, and prevented the oxidative degradation of doxorubicin by acting as an antioxidant. Optimal recovery was obtained by using a pH 4.5 ascorbate buffer of the described composition. Addition of the secondary amine ephedrine to the tissue prior to digestion improved recovery and reproducibility, probably by occupying irreversible adsorption sites present on the labware used, thereby preventing irreversible adsorption of other amines such as doxorubicin.

Before solid phase extraction of the digest, residual cell debris had to be removed from the resulting suspension in order to prevent clogging of the column. However, a major part of both doxorubicin and daunorubicin appeared to be adsorbed to this debris.

Therefore, prior to centrifugation, the drugs were first quantitatively removed from the solid material by vigorously shaking the suspension with methanol. Before extraction, the major part of the methanol had to be evaporated from the digest.

The solid-phase extraction procedure for doxorubicin from the tissue digest was identical to that for extraction of the drug from plasma [2]. Evidence exists that, apart from the expected weak interactions, strong (cat)ionic and polar interactions are operative in the solid phase extraction mechanism. As a result, an efficient, selective washing step of the sorbent was possible, resulting in very clean extracts free from any interfering substances (Fig. 1). The solid phase extraction of doxorubicin and daunorubicin, including the retention, washing and elution steps, was quantitative. The overall recovery, including digestion and solid phase extraction was limited only by the digestion step and amounted to 81.7 ± 2.1% (mean ±SD).

The proposed method has been implemented in a study of the pharmacokinetics and distribution kinetics of doxorubicin in the rat after different treatment regimens. Several hundred tissue samples have been processed and the method has proved to be very suitable for such studies in which large numbers of samples are involved.

References

[1] Q. G. C. M. van Hoesel, P. A. Steerenberg, D. J. A. Crommelin, A. van Dijk, W. van Oort, S. Klein, J. M. C. Douze, D. J. de Wildt and F. C. Hillen, *Cancer Res.* **44**, 3698–3705 (1984).
[2] G. de Groot, B. C. A. Tepas and G. Storm, *J. Chromatogr.* submitted.
[3] G. de Groot and K. L. Wubs, *J. Anal. Tox.* **11**, 175–178 (1987).
[4] S. Eksborg and H. Ehrsson, *J. Chromatogr.* **340**, 31–72 (1985).
[5] J. H. Beijnen, *Chemical stability of mitomycin and anthracycline antineoplastic drugs.* PhD-Thesis, University of Utrecht (1986).
[6] S. Eksborg and H. Ehrsson, *J. Pharm. Biom. Anal.* **2**, 297–303 (1984).
[7] M. J. H. Janssen, D. J. A. Crommelin, G. Storm and A. Hulshoff, *Int. J. Pharm.* **23**, 1–11 (1985).

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