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Short Communication

Determination of adriamycin in plasma and tissue biopsies

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ABSTRACT

A simple, rapid and sensitive method for the extraction and high-performance liquid chromatographic analysis of adriamycin in tissue and plasma is described. Tissue (5–100 mg) and plasma (1 ml) samples underwent a C_{18} Sep-Pak extraction into methanol. Chromatrography was performed on a μBondapak-phenyl column using a mobile phase of acetonitrile–0.1 *M* ammonium formate (pH 4.0) with a flow-rate of 2 ml/min. Fluorometric detection was used with an excitation of 480 nm and an emission of 550 nm. The procedure produced a linear curve for the concentration range 25–1000 ng/ml. The development of the assay produced rapid, repeatable and accurate results for both small tissue samples and plasma.

INTRODUCTION

Adriamycin is an anthracycline antibiotic used in the treatment of a variety of malignant diseases including leukemias, lymphomas and carcinomas of the breast, lung and ovary. Its clinical use is limited due to its cardiac toxicity [1–3]. Numerous studies have been conducted in man and animals to determine adriamycin pharmacokinetics with different treatment modalities to try and minimize its toxicity while maintaining therapeutic dosages.

Adriamycin extraction from plasma and its determination by high-performance liquid chromatography (HPLC) has been described by several groups [4–10]. These procedures involve the use of organic mixtures containing chloroform, hydrochloric acid–ethanol gels or C_{18} Sep-Pak extractions with a vacuum pump. There are a few methods involving the extraction of adriamycin from tissues or cells [10–19]. These methods use 1 g of tissue and involve lengthy extractions with saturated solutions and organic mixtures, which are then analyzed either by thin-layer chromatography, spectrofluorometry or HPLC.

This article describes a rapid and efficient C_{18} Sep-Pak clean-up of plasma and small tissue samples and adriamycin analysis using HPLC. This procedure allows rapid analysis of tissue biopsies of tumors and normal tissue that may facilitate

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evaluation of the potential for toxicity and therapeutic efficiency of dosage regimens.

EXPERIMENTAL

Reagents and standards

Acetonitrile and methanol were HPLC grade (Burdick & Jackson Labs., Muskegon, MI, U.S.A.). Ammonium formate, adriamycin and daunorubicin, the internal standard, were purchased from Sigma, (St. Louis, MO, U.S.A.). Sodium phosphate dibasic, reagent grade, was obtained from Mallinckrodt (Paris, KY, U.S.A.). The metabolite standards, adriamycinol, adriamycin aglycone and adriamycinol aglycone, were gifts from Adria Labs. (Columbus, OH, U.S.A.).

Stock standard solutions of adriamycin and daunorubicin were prepared by dissolving 5 mg each in 100 ml of HPLC-grade methanol. Working standards of each were then made by serial dilutions of stock standards with methanol. Standards were stable at −20°C in brown bottles for one month. Metabolite standards were also prepared by dissolution in methanol.

Apparatus

The analytical system consisted of a model 600E solvent delivery system, a Model 700 satellite WISP autosampler, an RCM 10 cm × 8 mm cartridge holder equipped with a 100 mm × 8 mm μBondapak-phenyl cartridge (10 μm particle size) and a CN Guard-Pak precolumn insert, a Model 470 scanning fluorescence detector and a NEC Powermate 2 computer system (Waters Assoc., Milford, MA, U.S.A.).

Chromatography

The mobile phase consisted of acetonitrile and 0.1 *M* formate buffer. The formate buffer was prepared from ammonium formate and adjusted to pH 4 with 4 *M* hydrochloric acid. The solvent was used in a linear gradient from 73% A (ammonium formate), 27% B (acetonitrile) to 70% A, 30% B up to 12 min and back to 73% A, 27% B at 18 min. The flow-rate was 2 ml/min and column temperature was ambient. Fluorescence detection of the peaks occurred with an excitation of 480 nm and an emission of 550 nm. All chromatograms were obtained with the following fluorometric conditions; gain 1000 ×, attenuation 1 and filter time constant 0.5 s.

Tissue extraction procedure

The tissue (5–100 mg) was placed in a Dounce glass tissue grinder (Baxter, McGraw Pk, IL, U.S.A.) and homogenized in 1.5 ml of 0.05 *M* sodium phosphate dibasic solution (pH 7.0) with ten to fifteen strokes. A 1-ml volume of the tissue/phosphate solution was transferred to a glass culture test tube and the internal standard daunorubicin (25 μl of a 5 μg/ml solution) was added to the

solution and vortex-mixed. This solution was added to a C_{18} Sep-Pak (Waters, Cat. No. 51910, used as a minichromatographic column) prewet with 3 ml of methanol, 3 ml of methanol–water (1:1) and 10 ml sodium phosphate dibasic (0.05 *M*, pH 7.0). The tissue/phosphate solution was introduced onto the Sep-Pak which was then washed with 3 ml of phosphate buffer and eluted with 3 ml of methanol. The methanol solution was placed in a warm water bath (45°C) and evaporated to dryness under nitrogen. The residue was then reconstituted in 500

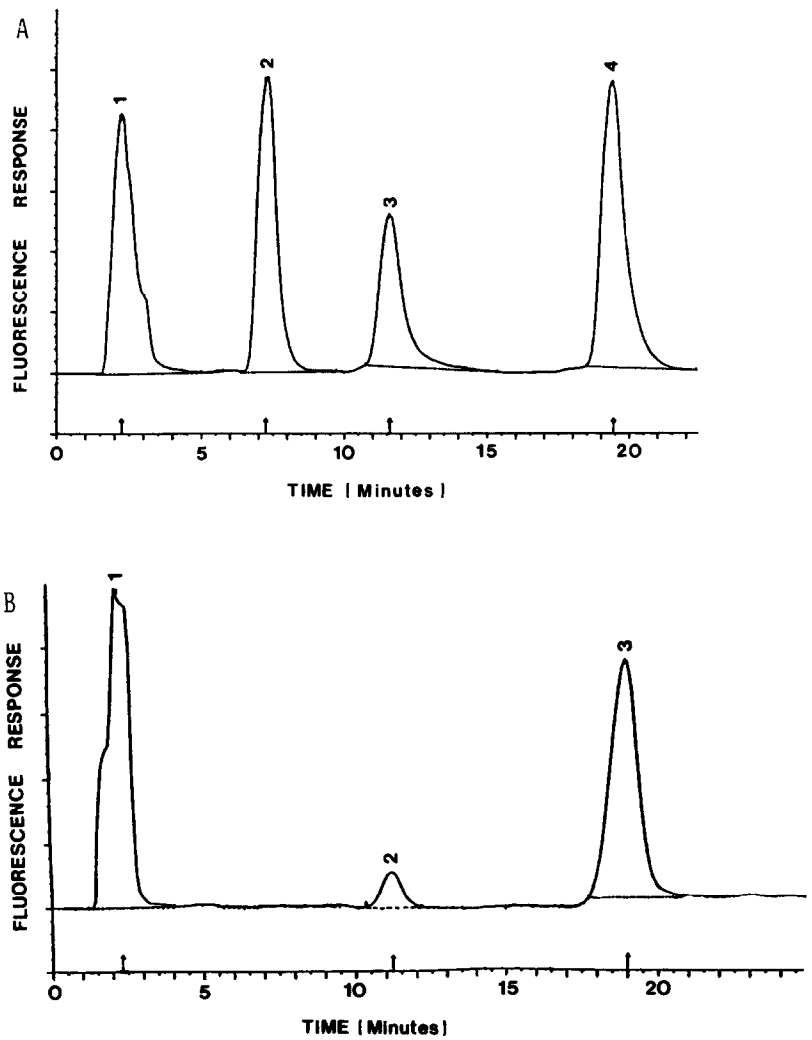

Fig. 1. (A) Chromatogram of a mixture of adriamycinol (2), adriamycin (3) and daunorubicin (4) in tissue (100 ng/5 mg spiked tissue). Peak 1 results from endogenous tissue components. (B) Chromatogram of a tissue sample collected 60 min after administration of 30 mg/m^2 adriamycin to a canine. Peaks: 1 = endogenous tissue components; 2 = adriamycin; 3 = daunorubicin.

μl of the mobile phase. The samples were briefly centrifuged (1000 *g* for 5 min) to remove any sediment and a 175-μl aliquot of the clear supernatant was injected into the liquid chromatograph.

Plasma extraction procedure

Previously frozen plasma samples were thawed and vortexed before use. Ap-

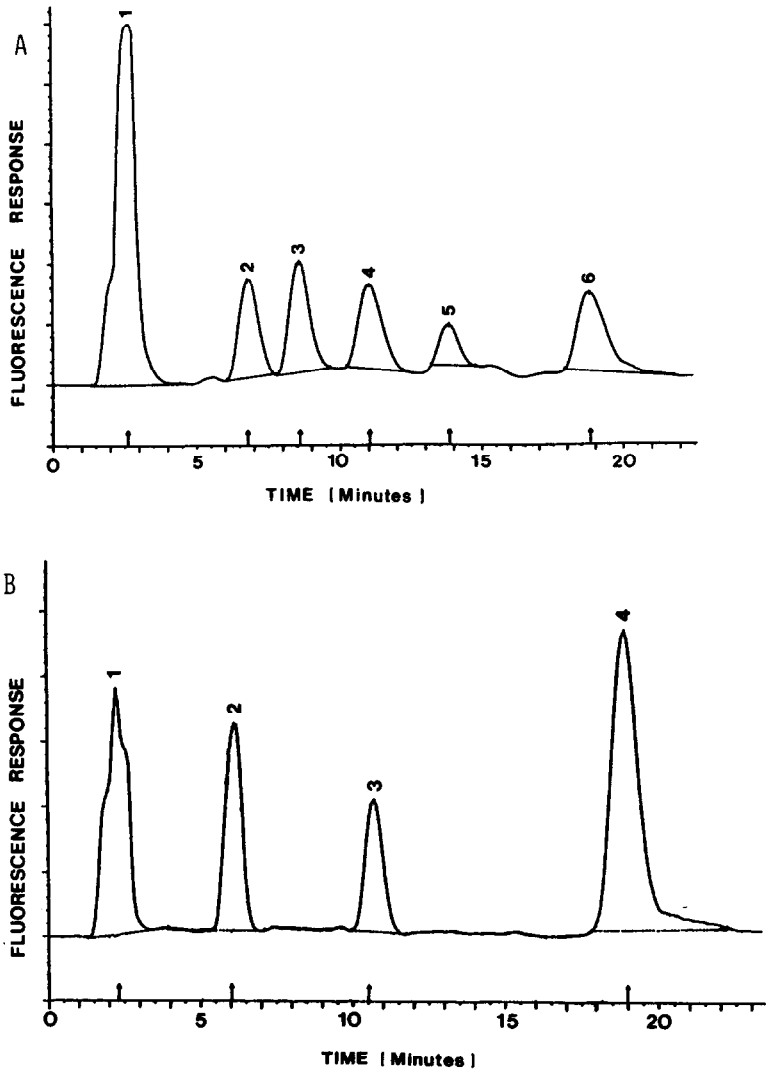

Fig. 2. (A) Chromatogram of a mixture of adriamycinol (2), adriamycinol aglycone (3), adriamycin (4), adriamycin aglycone (5) and daunorubicin (6) (100 ng/ml spiked plasma). Peak 1 results from endogenous plasma components. (B) Chromatogram of a plasma sample collected 15 min after administration of 30 mg/m^2 adriamycin to a canine. Peaks: 1 = endogenous plasma components; 2 = adriamycinol; 3 = adriamycin; 4 = daunorubicin.

propriate standard concentrations were evaporated under nitrogen to prevent methanol-induced protein precipitation upon addition of the plasma sample. The internal standard, daunorubicin (25 μl of a 5 μg/ml solution), was added and vortex-mixed. The plasma samples were then extracted using the method described for tissue.

RESULTS

A representative chromatogram for extracted tissue is shown in Fig. 1A. The retention times for adriamycin, adriamycinol and daunorubicin were 11.60, 7.26 and 19.44 minutes, respectively. The chromatogram in Fig. 1B is a canine tissue sample collected 60 min after a 30 mg/m^2 dose of adriamycin. Retention times for adriamycin and daunorubicin were 11.26 and 19.00 min, respectively. Endogenous peaks produced by the tissue did not appear after 2.25 min and, therefore, did not interfere with the elution of the anthracyclines. A representative chromatogram of the separation achieved in plasma is shown in Fig. 2A. Retention times for adriamycin, adriamycinol, adriamycin aglycone, adriamycinol aglycone and daunorubicin were 11.04, 6.79, 13.85, 8.60 and 18.33 min, respectively. Fig. 2B is a chromatogram of canine plasma collected 45 min after administration of 30 mg/m^2 adriamycin. Adriamycin, adriamycinol and daunorubicin retention times were 10.64, 6.05 and 19.01 min, respectively. Similar to tissue, peaks resulting from endogenous plasma components occurred prior to 2.62 min.

The method for tissue analysis produced a linear curve for the concentration range used in this study, with the correlation coefficients ranging from 0.998 to 0.999. Replicate analyses performed on the same day for tissues spiked with specific concentrations of anthracyclines showed coefficients of variation to be 7.1% for 30 ng/mg, 6.2% for 75 ng/mg and 7.6% for 150 ng/mg (Table I). Day-to-day variability for tissue replications are shown in Table II. The mean recoveries of adriamycin from tissue were 81, 85, 81, 87 and 80% for 10, 25, 50, 100 and 250 ng/mg, respectively. Adriamycin detection limit in tissue was 2 ng/mg. This would represent a peak approximately three times baseline noise.

TABLE I

WITHIN-ASSAY PRECISION OF ADRIAMYCIN FOR TISSUE STANDARDS (n = 4)

Concentration added (ng/5 mg tissue)	Concentration found (mean ± S.D.) (ng/5 mg tissue)	Coefficient of variation (%)
30	28.0 ± 2.5	7.1
75	72.2 ± 5.2	6.2
150	152.0 ± 12.4	7.6

TABLE II

BETWEEN-DAY ASSAY PRECISION OF ADRIAMYCIN FOR TISSUE STANDARD CURVE (n = 4)

Concentration added (ng/5 mg tissue)	Concentration measured (ng/5 mg tissue)	Area ratio[a] (mean ± S.D.)	Coefficient of variation (%)
10	8	0.02829 ± 0.0013	3.5
25	22	0.11232 ± 0.0016	2.0
50	43	0.21532 ± 0.0190	8.8
100	87	0.42355 ± 0.0202	4.7
250	148	1.09930 ± 0.0502	4.5

[a] Mean area ratio of adriamycin/internal standard.

Correlation coefficients for the linear plasma curve ranged from 0.995 to 0.999. Results of between-day replications for plasma are shown in Table III. The mean recoveries for adriamycin in plasma were 80, 82, 89, 73, 70, 75 and 90% for 25, 50, 100, 250, 500, 800 and 1000 ng/ml, respectively. The detection limit for adriamycin in plasma was 5 ng/ml. This represented a peak approximately three times baseline noise at the highest sensitivity on the fluorescence detector.

DISCUSSION

Pharmacokinetic and pharmacodynamic studies of adriamycin and its metabolites require analysis of both tissue and plasma. It is often impossible to remove large amounts of tissue from cancer patients or experimental animals, therefore,

TABLE III

BETWEEN-DAY ASSAY PRECISION OF ADRIAMYCIN FOR PLASMA STANDARD CURVE (n = 4)

Concentration added (ng/ml)	Concentration measured (ng/ml)	Area ratio[a] (mean ± S.D.)	Coefficient of variation (%)
25	20	0.24609 ± 0.011	4.5
50	41	0.35333 ± 0.031	8.8
100	80	0.71579 ± 0.053	7.4
250	182	1.81598 ± 0.058	3.2
500	365	3.64940 ± 0.254	7.0
800	586	9.22701 ± 0.524	5.7
1000	925	10.84006 ± 0.412	3.8

[a] Mean area ratio of adriamycin/internal standard.

an accurate reproducible procedure for small tissue biopsies is often needed. The procedure developed here has allowed the analysis of adriamycin tissue biopsies weighing less than 100 mg.

The detection limits and recoveries for both plasma and tissue samples are of equal sensitivity to existing methods for extraction and analysis of adriamycin. Reported plasma and tissue adriamycin concentrations in human patients and experimental animals range between 5 and 100 ng/ml. This method should be appropriate for patients and experimental animals administered therapeutic dosages of adriamycin.

The chromatograms do confirm the presence of an early peak due to endogenous plasma and tissue components. This did not interfere with the elution of adriamycin or its metabolites. There are different retention times for plasma and tissue samples. Theoretically they should be the same, but the different sample matrices caused a slight change in retention times.

The assay distinguished well between adriamycin and its most common metabolites, adriamycinol, adriamycinol aglycone and adriamycin aglycone, all of which have been identified in humans and animals [6,12,15,16]. This procedure has been used for analysis of canine samples collected from veterinary patients and experimental animals in our institution. Metabolites were detected only sporadically in the dog and have been described quantitatively in a paper by Wilke *et al.* [20]. The use of daunorubicin as an internal standard allows compensation for intra- and inter-assay variability in the extraction and chromatography steps.

Other HPLC procedures using similar extractions for plasma, such as described by Robert [4] or Van Lancker *et al.* [10], use either a chloroform–methanol or chloroform–isopropanol mixture for eluting the anthracyclines, while Yazigi and Saleh [7] attached a vacuum pump to the C_{18} Sep-Paks before eluting the samples. The use of a vacuum pump for large numbers of samples is time-consuming and its lack of use did not appear to affect our results. Methanol was found to work as well as or better than the chloroform–methanol or chloroform–isopropanol mixtures in the elution of the anthracyclines. This change eliminated the use of chloroform in the procedure. The extraction procedure for tissue used by Bolanowska and Gessner [11] used whole tissues and a chloroform–isopropanol (1:1, v/v) solution which was mixed with saturated sodium bicarbonate and sodium chloride. Bachur *et al.* [15], Mimnaugh *et al.* [12] and Cradock *et al.* [14] used 1 g of tissue and a chloroform–methanol (2:1, v/v) mixture in their extraction procedures. Anthracyclines were extracted from cell culture medium by Van Lancker *et al.* [10] using an ice-cold chloroform–isopropanol (4:1, v/v) mixture. Speth *et al.* [18] and Mahdadi *et al.* [19] extracted anthracyclines from human haematopoietic cells and rat hepatocytes using either a chloroform–methanol (9:1, v/v) or chloroform–butanol solution (1:1, v/v). Our procedure eliminated the use of organic mixtures and was accurate for 5–100 mg tissue samples.

In conclusion, a rapid, sensitive and clinically useful HPLC procedure has been developed for analysis of adriamycin in plasma and small tissue biopsies. This method would be appropriate for analyzing large numbers of samples.

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