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Note

New high-performance liquid chromatographic assay for plasma doxorubicin

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Doxorubicin (Adriamycin) is an anthracycline antibiotic employed in the treatment of a wide range of types of cancer. Its effectiveness is limited, however, by potentially severe myelosuppression and by total dose limiting cardiomyopathy [1, 2]. In an attempt to improve the drug's therapeutic index, long-term low-dose continuous infusion is currently being investigated as a method of treatment [3, 4]. This treatment requires an extremely sensitive assay, one capable of measuring doxorubicin in concentrations of 1 ng/ml or less in plasma. While assays utilizing fluorescence detection currently exist which can detect 1–2 ng quantities of doxorubicin, the use of only a fraction of the final processed plasma sample volume results in sensitivities in the range of 5—15 ng/ml of sample [5–7]. In addition, these assays often involve liquid–liquid extraction with organic solvents [5, 6] as well as other time-consuming sample handling techniques. A high-performance liquid chromatographic (HPLC) assay for daunorubicin and its metabolites has been devised using electrochemical detection [8]. This assay utilizes a loop column for sample extraction and has a sensitivity of 2 ng on column. However, the sample volume utilized was only 250 μl, resulting in a detection limit of 8 ng/ml of plasma.

We have developed an assay for doxorubicin that is rapid, simple to use, and highly sensitive; using electrochemical detection the detection limit is 2 ng/ml of plasma; with fluorescence detection 0.5 ng/ml doxorubicin in plasma is easily detectable. With this assay we are able to determine the levels of doxorubicin and doxorubicinol, a major metabolite of doxorubicin, in plasma samples taken from patients participating in a study of long-term low-dose continuous doxorubicin infusion [9]. Here we document the assay and report the levels measured.

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EXPERIMENTAL

Materials

Doxorubicin hydrochloride was obtained from Sigma (St. Louis, MO, U.S.A.). Doxorubicinol was kindly donated by Farmitalia (Milan, Italy), daunorubicin by Adria Labs. (Columbus, OH, U.S.A.). Ultraviolet (UV) grade acetonitrile was obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.), reagent-grade glacial acetic acid from J.T. Baker (Phillipsburg, NJ, U.S.A.). Tritiated water (0.25 mCi/g) and Aquasol liquid scintillation counter cocktail were obtained from New England Nuclear (Boston, MA, U.S.A.). All water used was obtained from a Waters Assoc. (Milford, MA, U.S.A.) Milli-Q water conditioning system.

Chromatography

The HPLC system consisted of an Altex Model 110 solvent metering pump, a Rheodyne Model 7125 sample injector valve equipped with a 400-μl sample loop, a 50 mm × 4 mm I.D. guard column filled with Waters Assoc. Bondapak Phenyl/Corasil packing (37–50 μm particle size), a 300 mm × 4 mm I.D. Waters Assoc. μBondapak Phenyl column (10 μm particle size), a Beckman Model 157 fluorescence detector and a BioAnalytical Systems Model LC-4 amperometric detector. Outputs from both detectors were recorded on a Scientific Products dual-pen recorder. The fluorescence detector was equipped with a 480-nm excitation filter (filter No. 096480) and a 560-nm emission filter (filter No. 096560). The electrochemical detector was equipped with a TL-5 thin-layer flow cell containing a glassy carbon electrode. The applied voltage was +0.700 V.

The mobile phase was an acetonitrile–acetic acid–water solution (27:1:72) adjusted to pH 4.3 with a 20% (w/v) sodium acetate solution. The solution was filtered with a Nuclepore 0.45-μm filter and degassed prior to use. A small portion of this mobile phase was set aside in a sealed container for use as an extraction buffer.

Flow-rate was 1.2 ml/min at a pressure of 48.3 bars.

Preparation of standards

The stock solution of 5 μg/ml doxorubicin was prepared in 0.1 m*M* hydrochloric acid containing 0.9% sodium chloride, stored in an aluminum foil covered glass container, and kept refrigerated at all times. The concentration of the stock solution was periodically checked by UV absorption measurements made at 233 nm and 253 nm in methanol using known molar extinction coefficients (ϵ_{233} = 38150 M^{-1} cm^{-1}, ϵ_{253} = 25500 M^{-1} cm^{-1}). Dilute solutions (< 50 ng/ml) of doxorubicin in water, methanol or ethanol were found to be unstable; solutions of <10 ng/ml of doxorubicin in hydrochloric acid–sodium chloride were found to be stable for >24 h when kept on ice in a dark environment. Doxorubicin standards in hydrochloric acid—sodium chloride for drug recovery experiments were prepared daily from the stock solution. Doxorubicin standards used for drug recovery experiments or for standard curve determinations were prepared weekly in plasma. Internal standard (daunorubicin) used in the assay was also prepared in plasma as described above.

Recovery data were obtained from a comparison of peak heights of samples of doxorubicin in hydrochloric acid–sodium chloride with peak heights of extracts of normal plasma spiked with equivalent amounts of doxorubicin.

Standard curves of peak height ratio of doxorubicin to daunorubicin versus doxorubicin concentration over the range 0–50 ng/ml were obtained by spiking 1-ml aliquots of normal plasma with varying amounts of doxorubicin and a constant amount of daunorubicin.

Cartridge preparation

The extraction cartridge consisted of a Rainin 200-μl disposable pipette tip filled with 50 mg of Waters Assoc. Bondapak Phenyl packing sandwiched between two glass wool plugs. The cartridge was attached to a Becton, Dickinson and Co. 10-ml plastic syringe.

Prior to use, the cartridge was activated by rinsing with 2 ml of extraction buffer (HPLC mobile phase), followed by a 4-ml water rinse.

Sample extraction

To each 1-ml sample to be analyzed, 5 ng of daunorubicin (internal standard, in 100 μl normal plasma) were added before extraction. The sample was then transferred to the syringe–cartridge combination and pushed through with the syringe plunger at a flow-rate of 0.3–0.5 ml/min, with the sample being collected in its original container. The sample was passed through the cartridge a second time at the same flow-rate, then discarded. The cartridge was washed once with 4 ml of water, after which 20 ml of air were pushed through the cartridge to remove as much of the water remaining in the cartridge as possible. Mobile phase buffer (300 μl) containing 30% acetonitrile (v/v) was then pushed through the cartridge at a flow-rate of 0.3–0.5 ml/min and collected in a polypropylene conical test tube. All of this extraction buffer was then immediately injected onto the column.

Extraction efficiency

To determine the percent recovery of the 300 μl of buffer used in the extraction procedure and the percent of the total applied to the HPLC column, 0.05 μCi of ^{3}H-labelled water was introduced into the extraction buffer, and aliquots were taken at several points in the extraction/injection process and analyzed for radioactivity. Samples were taken at the following points: (1) before doxorubicin was eluted from the cartridge; (2) after the eluate was collected; and (3) immediately prior to injection of the sample onto the column. The samples were collected in 10 ml of Aquasol liquid scintillation counter cocktail and analyzed on a Beckman Instruments Model LS 8100 liquid scintillation counter.

RESULTS AND DISCUSSION

Chromatography

Under the conditions used the elution order of compounds of interest was doxorubicinol, doxorubicin, and daunorubicin. The retention times were 6.6, 10.3, and 20.1 min, respectively. Fig. 1 shows a chromatogram of doxo-

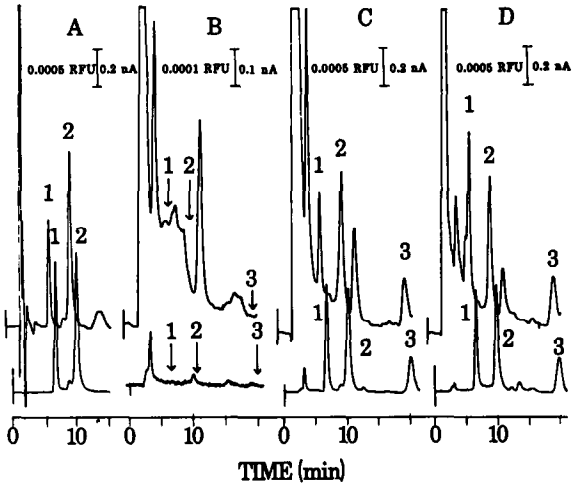

Fig. 1. Chromatograms of doxorubicinol, doxorubicin, and internal standard using electrochemical (top) and fluorescence (bottom) detection. (A) Doxorubicinol (10 ng) and doxorubicin (10 ng) standards; (B) drug-free human plasma; (C) human plasma spiked with 10 ng of doxorubicin, 10 ng of doxorubicinol, and 5 ng of internal standard; (D) plasma sample of patient 3 h after intravenous infusion of doxorubicin. Peaks: 1 = doxorubicinol, 2 = doxorubicin and 3 = daunorubicin, internal standard.

rubicinol and doxorubicin standards diluted in hydrochloric acid- sodium chloride and chromatograms of three plasma sample extracts; blank plasma, blank plasma spiked with doxorubicinol, doxorubicin, and internal standard; and plasma taken from a patient 3 h after an intravenous infusion of 70 mg of doxorubicin.

Extraction efficiency

In order to establish the efficiency of the extraction process, it is necessary to know the maximum attainable recovery (MAR) as well as the actual recovery. To determine the MAR, samples of the extraction buffer were analyzed at several points in the extraction/injection process for any concentration and/or volume changes that might affect the recovery of the drug. The results of the analyses show that: (1) the extraction buffer undergoes a 2.0 ± 0.4% dilution during the doxorubicin elution process; (2) only 90 ± 2% of the extraction buffer can be recovered from the cartridge; and (3) only 88 ± 7% of the cartridge eluate can be injected onto the column. If one assumes that the 2% dilution has only a negligible effect on the elution process and that the cartridge extracts 100% of the doxorubicin present in the plasma, then the MAR is 79%.

The percent of doxorubicin recovered by the extraction process was established by determining the recovery of varying amounts of doxorubicin from a 1-ml plasma sample and the recovery of a fixed amount of doxorubicin (10 ng) from various-sized samples. Table I lists these results, which show that the percent of doxorubicin recovered varies from 66.1 ± 0.9% to 70.0 ± 1.4% depending on sample volume, while recovery from 1 ml of plasma is essentially independent of doxorubicin concentration at 69.7 ± 0.3%. The extraction process therefore has an efficiency of 0.88.

TABLE I

MEAN PERCENT RECOVERY OF DOXORUBICIN FROM PLASMA (n = 3)

Amount of doxorubicin (ng)	Sample volume (ml)	Percent recovery (± S.D.)
1	1.0	69.6 ± 0.7
10	1.0	70.0 ± 0.4
100	1.0	69.6 ± 0.6
10	0.5	67.4 ± 1.0
10	2.0	67.6 ± 1.5
10	3.0	66.1 ± 0.9

Recovery of doxorubicin from 1 ml of plasma using only a single sample pass through the cartridge, as well as using three sample passes through the cartridge, was briefly examined. For a 1-ml plasma sample containing 10 ng of doxorubicin it was found that using a single sample pass through the cartridge reduced the recovery to 52.7 ± 3.0% while increasing the number of sample passes to three did not significantly increase the recovery. Moreover, the cartridge exhibited a tendency to clog during the third pass. Plasma samples larger than 3 ml also tended to clog the cartridge when extracted with only two sample passes.

Recovery of doxorubicin from 1 ml of plasma as a function of amount of packing used was also examined. Recoveries from 25 mg of packing were 9.1 ± 0.8% less than with 50 mg; recoveries with 100 ng were 8.6 ± 1.3% greater than with 50 mg. However, at 100 mg, clogging of the cartridge occurred quite frequently even on the first pass of a 1-ml sample.

Quantitation of standards

A plot of doxorubicin added to plasma versus doxorubicin measured in plasma was prepared from standard curve data gathered over several weeks for each method of detection. For fluorescence detection the slope was 1.004, the y-intercept was 0.02, and the correlation coefficient was 1.00. For electrochemical detection the slope was 1.01, the y-intercept was 0.03, and the correlation coefficient was 1.00. Sensitivity with the electrochemical detector has a lower limit of 2 ng per sample; with the fluorescence detector 0.5 ng per sample levels are easily determined.

Doxorubicinol

Quantitation of doxorubicinol, one of the major metabolites of doxorubicin, was also examined. The recovery and fluorescence detection limits of doxorubicinol are similar to those of doxorubicin. The electrochemical detector is approximately 40% more sensitive to doxorubicin than to doxorubicinol, resulting in a proportionately higher detection limit for doxorubicinol.

Application

We have used the HPLC assay described here to analyze serial plasma samples from a patient with metastatic adenoid cystic carcinoma of the parotid. This patient was part of a phase I study to examine the safety of long-term low-dose continuous doxorubicin infusion therapy using an implanted Medtronic pump.

Details of the study are published elsewhere [9]. Briefly, infusion of doxorubicin was initiated at a dose of 1.5 mg/m^2/day, and in the absence of severe symptoms of toxicity, the dose was escalated by 0.5–0.75 mg/m^2/day every two weeks. Plasma samples were taken every two weeks prior to dose escalation. The samples were collected in heparinized tubes, centrifuged, and frozen at −20°C for two to four weeks prior to the analysis for doxorubicin and doxorubicinol using fluorescence detection. Results are listed in Table II. Doxorubicin and doxorubicinol plasma concentrations increased in a dose-dependent manner. On four out of five days the ratio of doxorubicin to doxorubicinol concentration appeared independent of dose, remaining at approximately 2.

TABLE II

DOXORUBICIN AND DOXORUBICINOL PLASMA CONCENTRATIONS IN A PATIENT RECEIVING LOW-DOSE CONTINUOUS DOXORUBICIN INFUSION

Sample date	Dose* (mg/day)	Doxorubicin concentration (ng/ml)	Doxorubicinol concentration (ng/ml)
14	2.59	2.0	0.8
28	3.21	1.4	1.3
56	5.64	7.2	3.5
68	6.25	8.4	4.4
82	6.21	9.9	5.2

*Dose listed is for the two-week period prior to sample date; dose administered was based on patient's body surface area (1.58 m^2).

The assay described here is rapid, highly sensitive, and easy to use. It replaces the widely used multiple liquid–liquid extractions with a single solid–liquid extraction, and results in a concentration of sample in a final volume small enough to be totally analyzed. This procedure eliminates the need for concentrating the final sample by evaporation, or analysis of only a small fraction of the final sample volume. Utilization of the entire final sample volume yields sub-nanogram sensitivity, which allows the method to be used to measure heretofore undetectable plasma levels of doxorubicin and doxorubicinol in patients undergoing low-dose continuous doxorubicin infusion, as well as allowing its use with patients undergoing standard treatment.

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