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MEASUREMENT OF ADRIAMYCIN (DOXORUBICIN) AND ITS METABOLITES IN HUMAN PLASMA USING REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND FLUORESCENCE DETECTION

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SUMMARY

We describe a method for measuring adriamycin and its major metabolite, adriamycinol, in plasma, using reversed-phase high-performance liquid chromatography and fluorescence detection. The lower limit of detection is approximately 1 ng/ml for both compounds; within-day coefficients of variation are 3.6% and 4.4% for adriamycin and adriamycinol, respectively. A slight modification of this procedure also allows measurement of aglycone metabolites.

INTRODUCTION

Adriamycin (Ad, doxorubicin) is an anthracycline antibiotic which has been used in the treatment of a variety of neoplastic diseases in man [1, 2]. In the study of the pharmacokinetics of this drug, several methods for measurement of adriamycin and its metabolites in biological samples have been used, including total extractable plasma fluorescence [3, 4], thin-layer chromatography (TLC) followed by elution and fluorescent measurement [5], TLC with quantitative fluorescent scanning of the thin-layer plate [6], and radioimmunoassay (RIA) [7]. High-performance liquid chromatography (HPLC) with a diphenyl bonded column has been used by Langone et al. [8] as a preparatory step for separating fractions prior to RIA. Israel et al. [9] have applied adsorptive and reversed-phase (diphenyl bonded) chromatography to the measurement of adriamycin and its metabolites in rabbit bile using both ultraviolet and fluorescence detection. Using ultraviolet and visible light detection, Eksborg [10] has studied the separation of adriamycin, daunomycin, and their hydroxyl metabolites under various conditions, but he did not evaluate biological fluids. We

describe a sensitive reversed-phase (ODS column) HPLC procedure, employing fluorescence detection for the separation and quantitation of adriamycin, adriamycinol, and their respective aglycones, in human plasma. The closely related compound daunomycin (Da) and its aglycone derivative (daunomycinone) are used as internal standards.

MATERIALS AND METHODS

Apparatus

We initially used a Perkin-Elmer dual pump high-pressure liquid chromatograph (Model 601) equipped with a Rheodyne injector valve (Model 7120) with a 20-μl loop and a Model 204-S Perkin-Elmer fluorescent detector. For later work a Perkin-Elmer (Series 2) high-performance liquid chromatograph and a Perkin-Elmer Model 650-10LC fluorescent detector were used, with comparable results. The column ultimately used (25 × 0.26 cm) had a C_{18} (5-μm) reversed-phase packing, ODS "Hi-Eff." (Applied Science Labs., State College, Pa., U.S.A.). In preliminary work other columns evaluated were C_{18} (10 μm): Partisil 10/25 ODS and Partisil 10/25 ODS-2 (Whatman, Clifton, N.J., U.S.A.).

Reagents and standards

Adriamycin HCl was a gift of Adria Laboratories (Columbus, Ohio, U.S.A.) and adriamycinol HCl was a gift of Farmitalia (Milan, Italy). Daunomycin HCl was obtained from Sigma (St. Louis, Mo., U.S.A.) and acetonitrile "Distilled in Glass" from Burdick and Jackson Labs. (Muskegon, Mich., U.S.A.). Other reagents and drugs used for interference studies were obtained from usual commercial sources.

Stock solutions of adriamycin, adriamycinol, and daunomycin were made in methanol (concentration 100 μg/ml) and stored at —20°. Aglycones of all of these drugs were made as previously described [2] by mild acid hydrolysis (0.1 *M* HCl at 55° for 45 min) followed by neutralization, extraction of the aglycone into chloroform, evaporation and redissolution of the residue in methanol. Purity was checked by TLC [2] and by HPLC. The concentrations of the aglycone solutions were calculated, based on the molar extinction of daunomycinone [11] and the equivalent molar extinction for the aglycones of adriamycin and adriamycinol [2, 12]. Concentrations for daunomycin and daunomycinone internal standard solutions were 2 μg/ml, in methanol.

Plasma standards were made by supplementing blood bank plasma with stock solutions of each of the drugs to final concentrations of 2—200 ng/ml. Aliquots were protected from light and stored frozen at —20°.

Plasma samples from patients receiving adriamycin were collected in heparin. The plasma was separated and stored in the dark at —20° until assayed within one week. Samples for interference studies were obtained from patients not receiving adriamycin. These were chosen to include samples from patients with renal failure and hepatic disease, as well as samples from patients receiving cancer chemotherapy other than adriamycin, and samples with in vitro hemolysis.

Extraction procedure

Daunomycin, the internal standard (50 μl, 100 ng) was added to each 2.00-

ml sample and mixed in a 15-ml PTFE-lined screw-top glass test-tube. Following alkalinization with 0.1 ml of 0.2 *M* sodium hydroxide the sample was extracted with 10 ml chloroform—isopropanol (2 : 1) for 10 min. After centrifugation (1000 *g* for 10 min) the upper aqueous layer was removed by aspiration to facilitate transfer of the lower organic layer to a standard test tube. After drying with anhydrous sodium sulfate the organic layer was transferred to a tapered 15-ml centrifuge tube and evaporated in a water-bath at 40° under an air stream. During evaporation the sides of the tube were washed with chloroform or chloroform—methanol (1 : 1) in order to increase recovery. The residue was dissolved in 50—100 μl of methanol, and 10—20 μl were submitted to chromatography.

The extraction procedure for the aglycones was identical except that the internal standard used was 50 μl (100 ng) of daunomycinone.

Chromatographic procedure

The mobile phase consisted of 50% acetonitrile (by volume) in 50% 0.01 *M* phosphoric acid (pH 2.3). The flow-rate was 1 ml/min, and column temperature was maintained at 25°. The fluorometer was set at 465 nm excitation wavelength (slit width 10 nm) and 580 nm emission (slit width 20 nm). Quantitation was based on peak height ratios.

For measurement of aglycones, the percentage of acetonitrile in the mobile phase was decreased to 36—40%; otherwise, the procedure was identical.

To exclude drug interferences, methanolic solutions of drugs were injected in quantities greater than would be expected in 2 ml of plasma.

RESULTS

Standard curves based on peak height ratios for adriamycin and adriamycinol were both linear ($r = 0.99$) in the ranges 0—100 ng/ml and 0—50 ng/ml, respectively. The analysis of ten samples, each containing 10 ng/ml adriamycin and 5 ng/ml adriamycinol, showed within-day coefficients of variation of 3.6% and 4.4%, respectively. Retention times were: daunomycin, 18 min ($k = 4.7$); adriamycin, 11.4 min ($k = 2.44$); adriamycinol, 8.7 min ($k = 1.45$); adriamycinone, 5.4 min ($k = 0.72$); adriamycinol aglycone, 4.2 min ($k = 0.36$) (see Fig. 1). Extraction of many plasma and serum blank samples resulted in peaks with retention times of 2.7—5.2 min (Fig. 2); therefore, to obtain better resolution of early eluting peaks, the procedure was modified for aglycones by reducing the proportion of acetonitrile to 36—40%. Using 40% acetonitrile, retention times were: daunomycinone, 19 min ($k = 4.8$); adriamycinone, 9 min ($k = 1.6$); adriamycinol aglycone, 6.4 min ($k = 0.67$); and all peaks in drug-free plasma eluted between 3 and 6 min (Fig. 3). Using these modified conditions and daunomycinone as an internal standard, linear standard curves were obtained for adriamycinone (range 0—20 ng/ml, $r = 0.99$) and adriamycinol aglycone (range 0—40 ng/ml, $r = 0.99$). Analysis of ten replicate samples revealed coefficients of variation of 5.1% (adriamycinone) and 4.0% (adriamycinol aglycone). Recoveries for the parent drug, the metabolites, and the two internal standards were in the range 65—75%. These were corrected by the use of plasma standards and the use of an internal standard carried through the entire extraction

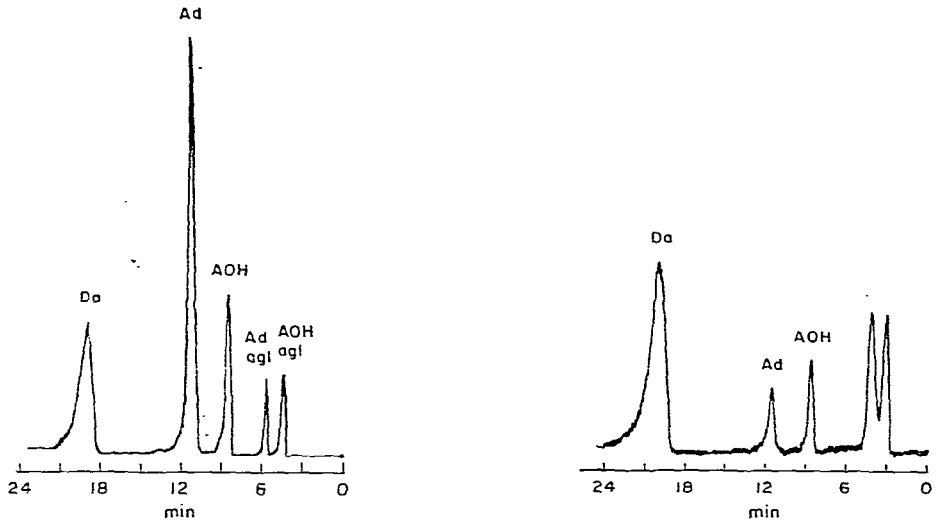


Fig. 1. Mixture of 5 drugs: daunomycin (Da), adriamycin (Ad), adriamycinol (AOH), adriamycinone (Ad agl), and adriamycinol aglycone (AOH agl). Chromatographic conditions: 50% acetonitrile, 50% 0.01 *M* phosphoric acid, flow-rate 1 ml/min, temperature 25°. Fluorescence detection: excitation wavelength 465 nm, emission 580 nm.

Fig. 2. Plasma sample supplemented with 5 ng/ml adriamycin (Ad) and 5 ng/ml adriamycinol (AOH); internal standard 50 ng/ml daunomycin (Da). Chromatographic conditions as in Fig. 1. Unidentified peaks (3—5 min) were present in chromatograms from all plasma or serum samples tested.

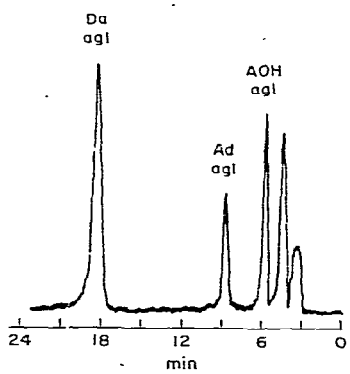


Fig. 3. Plasma sample supplemented with 7.8 ng/ml adriamycinone (Ad agl) and 7.6 ng/ml adriamycinol aglycone (AOH agl); internal standard 50 ng/ml daunomycinone (Da agl). Chromatographic conditions: 40% acetonitrile, 60% 0.01 *M* phosphoric acid, flow-rate 1 ml/min, temperature 25°.

procedure. Careful temperature control during the solvent evaporation step of the extraction procedure was critical; if the temperature exceeded 45° these drugs, including adriamycin, its metabolites, and both internal standards, might decompose.

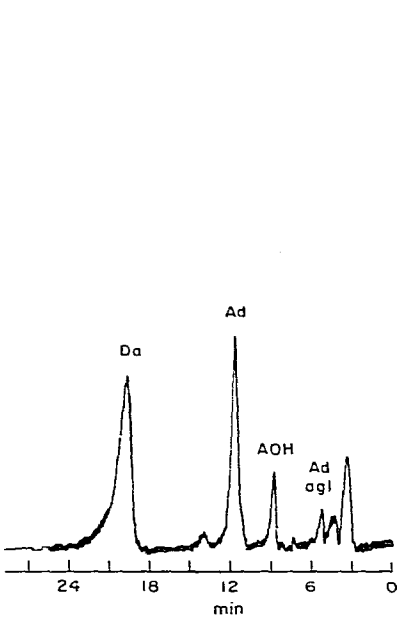
Analysis of forty commonly used drugs, including naturally fluorescent compounds, analgesics, tranquilizers, and sedatives, revealed no interfering peaks

(see Table I). However, methyldopa and oxycodone eluted at 6 min (using 50% acetonitrile), which was slightly later than adriamycinone, 5.4 min.

TABLE I

DRUGS TESTED FOR INTERFERENCE WITH MEASUREMENT OF ADRIAMYCIN AND ITS METABOLITES

Acetaminophen	Meperidine, normeperidine
Allylisobutylbarbital	Methadone
Amitriptyline	Methaqualone
Amobarbital	Methyldopa
Aspirin	Morphine
Caffeine	Nitroprusside
Chlordiazepoxide	Nortriptyline
Chlorpromazine	Pentazocine
Codeine	Pentobarbital
Desipramine	Phenobarbital
Diazepam, nordiazepam	Procainamide, N-acetylprocainamide
Diphenhydramine	Propoxyphene
Dopamine	Propranolol
Furosemide	Protriptyline
Hydroxyzine	Quinidine
Imipramine	Secobarbital
	Theophylline



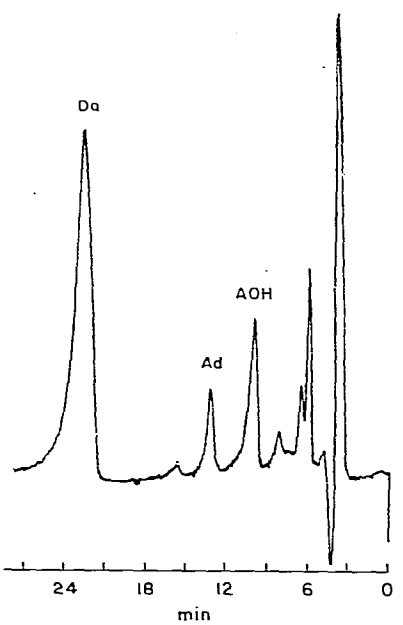


Fig. 4. Sample from patient who received 45 mg adriamycin 2 h previously. Chromatographic conditions as in Fig. 1. Peaks represent: Ad, adriamycin (25 ng/ml); AOH, adriamycinol (5.5 ng/ml); Ad agl, adriamycinone (1 ng/ml). Internal standard is Da, daunomycin (50 ng/ml).

Fig. 5. Sample from patient who received 30 mg adriamycin 15 h previously. Chromatographic conditions as in Fig. 1. Peaks represent: Ad, adriamycin (4.0 ng/ml); AOH, adriamycinol (4.8 ng/ml). Peak corresponding to adriamycinone coelutes with plasma peaks. Internal standard is Da, daunomycin (50 ng/ml).

Samples from patients not receiving adriamycin were analyzed by the same procedure and showed no interferences for adriamycin, adriamycinol, daunomycin, or daunomycinone. When analyzed by the modified procedure for aglycones, samples from two patients (both patients receiving multiple drugs including methyldopa) resulted in several minute peaks coeluting with adriamycinone and adriamycinol aglycone; these peaks corresponded to less than 2 ng/ml of either aglycone equivalent.

A chromatogram from a patient receiving adriamycin is illustrated in Fig. 4. The major peaks correspond to adriamycin, adriamycinol, and adriamycinone. Fluorescent scanning of each of these three peaks was performed on a subsequent sample by stopped flow technique. The resultant activation and emission scans were all identical with those obtained from the authentic compounds under the same conditions. Figs. 5 and 6 show chromatograms from the plasma of a second patient who had received 30 mg of adriamycin 15 h previously. Using 50% acetonitrile (Fig. 5), peaks correspond to 4.0 ng/ml adriamycin and 4.8 ng/ml adriamycinol; but peaks which would correspond to aglycones are not resolved from unidentified plasma components. Reduction of the acetonitrile concentration to 40% (Fig. 6) improves the resolution of these early peaks, with adriamycinone 7 ng/ml, and adriamycinol aglycone less than the limit of detection for this assay (<1 ng/ml). Fig. 7 shows a chromatogram from

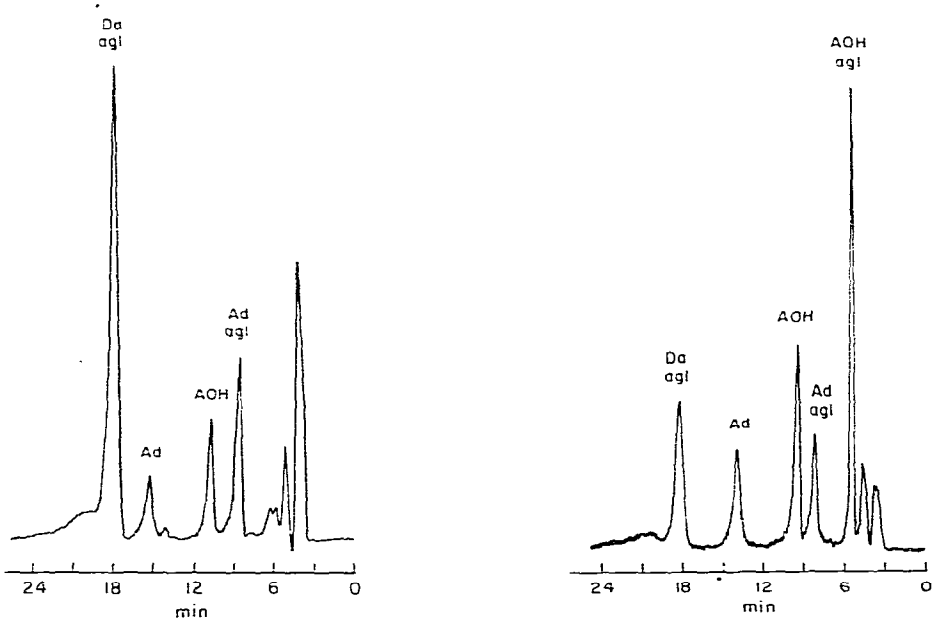


Fig. 6. Sample from the same patient as in Fig. 5. Chromatographic conditions modified; 40% acetonitrile, 60% 0.01 *M* phosphoric acid, flow-rate 1 ml/min, temperature 25°. Peaks: Ad, adriamycin; AOH, adriamycinol; and Ad agl, adriamycinone (7 ng/ml). Internal standard is Da agl, daunomycinone (50 ng/ml).

Fig. 7. Sample from patient who received 96 mg of adriamycin 17 h previously. Chromatographic conditions as in Fig. 3 (modified method). Major peaks: Ad, adriamycin (20 ng/ml); AOH, adriamycinol (21 ng/ml); Ad agl, adriamycinone (12 ng/ml) and AOH agl, adriamycinol aglycone (29 ng/ml). Internal standard is Da agl, daunomycinone (50 ng/ml).

another subject who had received 96 mg of adriamycin 17 h previously. In this instance adriamycin and relatively high concentrations of adriamycinol and aglycone metabolites are present. Since this chromatogram was run under modified conditions (40% acetonitrile) daunomycinone is the internal standard; daunomycin is not present.

DISCUSSION

Pharmacokinetics of adriamycin and its metabolites have been studied in man [4, 5, 13, 14], rabbits [15], mice and rats [9, 16], monkeys [7], as well as in enzyme systems [17]. The major metabolite in humans and in other species studied is adriamycinol, though eight metabolites in the urine [13] and eleven metabolites in bile [18] have been reported. Since both adriamycin and adriamycinol have cytotoxic effects [19], it would be desirable to measure both of these compounds. Assays based on total extractable fluorescence measure the parent drug and fluorescent metabolites without discrimination. Additionally, it is reported that total plasma extractable fluorescence may be elevated due to the presence of certain steroids or bile acids [14] causing interference, particularly at low drug levels. For the separate quantitation of metabolites the fluorescent assays have been used in conjunction with chromatographic separation, most commonly using TLC. RIA measures adriamycin and adriamycinol equally, and cross reacts with the aglycones to a lesser degree [7]. The use of HPLC for separation, followed by RIA for quantitation, has been described [8].

Eksborg [10] has studied systematically the use of reversed-phase HPLC for separation of adriamycin and daunomycin, and their hydroxyl metabolites, with ultraviolet or visible light absorbance spectrophotometric detection. However, because fluorescence detection has the potential for both increased sensitivity and specificity, we have selected this mode. Our results indicate sensitivity to drug levels less than 2 ng/ml, and interferences from common drugs tested are negligible. The sensitivity and linearity range are appropriate for measurement of the levels of adriamycin and adriamycinol expected following the usual therapeutic dose of this drug.

In preliminary work several hydrocarbon reversed-phase columns were tried. Columns with 10-μm particle size packing did not provide sufficient separation and yielded broad peaks. The use of 5-μm packing resulted in substantial improvement in resolution and peak configuration.

For measurement of adriamycin and adriamycinol, daunomycin was used as an internal standard; it has both structural similarity and an identical fluorescent spectrum with these two drugs. For the smaller but less water soluble aglycones, daunomycinone was used as an internal standard. These two internal standards were not compatible with use in a single extraction. Using chromatographic conditions optimized for adriamycin (50% acetonitrile), daunomycinone would coelute with adriamycinol; conversely, if conditions were optimized for measurement of the aglycones (36—40% acetonitrile), retention of daunomycin was 30 min.

We evaluated this procedure on several patients who had received adriamycin at various times before blood sampling. Adriamycin, adriamycinol, and ap-

parent levels of adriamycinone were found in all patients. The third patient (Fig. 7), from whom the sample was obtained 17 h after medication, showed peaks corresponding to 12 ng/ml of adriamycinone and 29 ng/ml of adriamycinol aglycone, in addition to the parent compounds. All these peaks showed fluorescent emission and activation scans identical to the authentic material. We cannot of course verify that in vitro degradation did not contribute to the presence of these aglycones. However, supplemented plasma standards showed no detectable in vitro degradation under identical conditions; although, if kept at room temperature for 36 h, adriamycin and adriamycinol showed degradation corresponding to 34% and 11%, respectively. Although very little of the unconjugated aglycones has been thought to occur in plasma [4, 6], Riggs et al. [18] and Watson and Chan [6] have detected the presence of aglycone metabolites in human plasma using TLC.

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