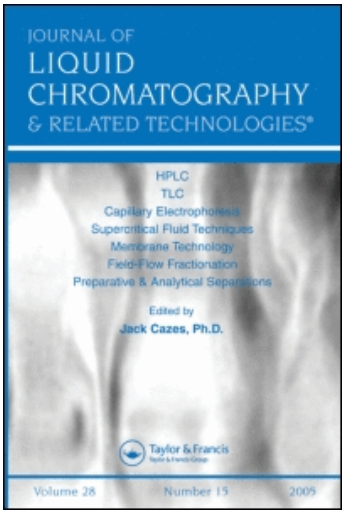

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Liquid Chromatographic Analysis of Adriamycin and Metabolites in Biological Fluids

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LIQUID CHROMATOGRAPHIC ANALYSIS OF ADRIAMYCIN AND METABOLITES IN BIOLOGICAL FLUIDS

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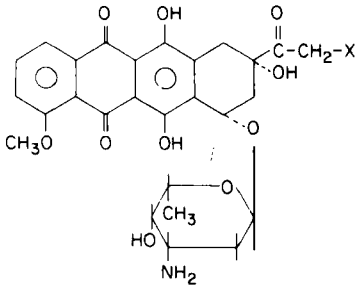
ABSTRACT

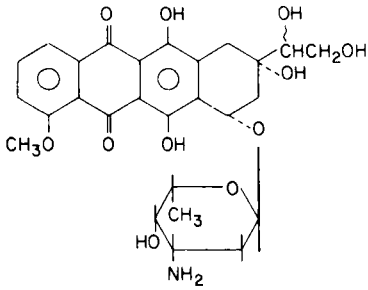
A definitive approach to the analysis of plasma, bile, and urine levels of the widely used antitumor drug Adriamycin using two complementary high performance liquid chromatographic systems, one normal phase and the other reversed phase, is described. Sensitivity in the 2-10 picomole per sample range is achieved by means of fluorescence detection. The use of the two systems in parallel provides an unequivocal basis for the characterization and determination of Adriamycin and its principal metabolite, adriamycinol, and enables the measurement of anthracycline fluorescent aglycones and conjugates, as well.

INTRODUCTION

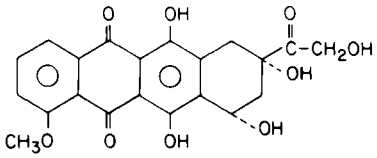
Adriamycin (ADR) and daunorubicin (Fig. 1) are structurally-related anthracycline antitumor antibiotics derived from Streptomyces fermentations. While daunorubicin shows a very narrow spectrum of antitumor activity, ADR is widely used clinically in the treatment of leukemias and various solid tumors (1,2). Both agents produce acute hematologic and delayed cardiac side-effects which are dose-limiting. For several years our laboratory has been engaged in studies to extend the therapeutic effectiveness of ADR (3) and to design potentially superior ADR analogs (4-7). Such studies require a detailed understanding of ADR pharmacology



Daunorubicin, X=H
Adriamycin, X=OH



Adriamycinol



Adriamycinone

FIGURE 1

Structures of daunorubicin, Adriamycin (ADR), adriamycinol (AMNOL), and the ADR aglycone, adriamycinone.

and mechanism(s) of action. Towards this end, a means of measuring fluid, tissue, and cellular levels of parent drug and metabolites, and of monitoring the routes and rates of excretion of these various components, has been essential.

A number of analytical approaches have been described for estimating anthracyclines in biological systems. These methods, however, all suffer from one or more deficiencies. Thus, direct radioimmunoassay (RIA) (8), and the measurement of total fluorescence (especially after acidic ethanol treatment) (9), are reasonable methods for quantifying total anthracycline content in plasma, but neither will differentiate parent drug from metabolites. Reverse phase high performance liquid chromatography (HPLC), coupled with RIA, has been reported (10); but the method,

as described, is lengthy and tedious, with each sample requiring 30 minutes for HPLC separation, followed by lyophilization of up to 30 fractions and quantitation by RIA, a process which may not be equally sensitive for different metabolites.

The most widely used technique has been thin layer chromatography (TLC) on silica gel, both for qualitative and quantitative purposes. Direct fluorescence scanning (11-13), and elution of scrapings from TLC plates, followed by fluorescence measurement (14-17), have been reported. However, the light- and air-sensitivity of the anthracycline chromophore can lead to underestimation by these methods, due to chromophore bleaching prior to fluorescence analysis. Furthermore, and of greater significance, the acid-sensitivity of the glycosidic linkage can result in the formation of aglycone artifacts during TLC because of the acidic nature of silica gel. Using liquid chromatographic conditions previously developed in our laboratory (6,7), we have shown, for example, that pure ADR standards, free of aglycone by HPLC, exhibit measurable and significant quantities of adriamycinone, the ADR aglycone (Fig. 1), after development on silica gel TLC plates with chloroform-methanol-water solvent systems; greater amounts of adriamycinone are produced when plates are developed with acetic acid-containing solvent mixtures, as commonly employed by others (18). Recently, Hulhoven and Desager reported extraction and HPLC conditions which allow a more direct quantitative determination of low levels of daunorubicin in plasma using visible absorption detection (19,20).

We now describe our improved analytical schema for ADR and metabolites utilizing complementary HPLC assay systems, one normal phase and the other reversed phase, with flow fluorescence detection. The sampling and analytical methods are rapid, reproducible, and highly sensitive, and are applicable to the determination of anthracyclines in plasma, bile, and urine. The use of the two systems in parallel allows for the unambiguous characterization of ADR and its principal metabolite, adriamycinol (AMNOL).

MATERIALS AND METHODS

Chemicals

Bulk ADR hydrochloride, kindly provided by Farmitalia S.p.A., Milan, Italy, and AMNOL and adriamycinone, prepared in this laboratory according to previously reported procedures (21,22), were used as reference standards. For animal and human use, clinical grade ADR, containing lactose excipient, was formulated in sterile 0.9% saline for injection U.S.P.

Extraction solvents were certified Fisher reagent grade. Chloroform, methanol, and acetonitrile, used for the HPLC separations, were "Distilled-in-Glass" solvents obtained from Burdick and Jackson Laboratories Inc., Muskegon, Mich. Acetic acid, formic acid, and aqueous ammonia were certified Fisher laboratory grade reagents. Filtered deionized water, used directly and in preparation of buffers, was obtained from a Milli-Q water purification system (Millipore Corp., Bedford, Mass.). Aqueous pH 4.00 buffer was 0.1% (w/v) ammonia in deionized water adjusted to pH 4.00 with 97% formic acid.

Instrumentation

For normal phase liquid chromatography a Waters Associates Model ALC/202 liquid chromatograph fitted with a 25 cm x 4.6 mm ID prepacked Whatman Partisil-10 PAC modified normal phase column (cyano-aminoalkyl derivative of silica gel) was used. Signal detection was by means of a Schoeffel Instrument Co. Model SF-970 flow fluorescence detector, with the excitation wavelength set at 482 nm (deuterium lamp energy source). An emission filter (Schoeffel #2-73), with low wavelength cut-off near 550 nm was used. The optimal developing system was a programmed gradient of a four component mixture of chloroform, methanol, acetic acid, and water, in the ratio 850:150:50:15 (Solvent B), mixed with chloroform (Solvent A). A Waters Associates solvent programmer, Model 660, was set to run for 2 minutes at 2 ml/min on profile #4 (early rapid change followed by slow finish) with initial condi-

tions set at 10% solvent B and final conditions 100% B. The column was eluted at final conditions for at least 6 minutes. Reequilibration times at initial conditions were of the order of ten minutes or longer to insure reproducible retention times. Operating pressures did not exceed 1200 psig.

For reverse phase HPLC a Waters Associates Model ALC/244 liquid chromatograph fitted with a prepacked Waters μ-Bondapak/phenyl 30 cm x 3.9 mm ID column and a Schoeffel SF-970 fluorometer were used. A Waters Associates Model 660 solvent programmer was set to run for 5 minutes at 3.5 ml/min on a linear program. Initial conditions were 30% acetonitrile (containing 2% aqueous buffer for proper pump operation) (Solvent B) and 70% aqueous pH 4.00 ammonium formate buffer (Solvent A). Final conditions were 35% B, 65% A. Reequilibration times of 5 to 6 minutes were adequate for reproducible retention times. Operating pressures with this system were just over 2000 psig with new columns; the high flow rate aids in peak sharpening.

Sample Preparation

Plasma. Plasma samples (1.0 ml) were adjusted to pH 8.5 with Tris buffer and were diluted with 1.5 ml of methanol. Precipitated proteins were removed by centrifugation at 25,000 x g for 20 minutes at 5°. The supernatant was extracted three times with 2 ml portions of chloroform in glass-stoppered conical centrifuge tubes equipped with silicone rubber septa at the bottom (Kontes Glass Co., Vineland, New Jersey). Each extraction was followed by centrifugation at 1000 x g for 5 minutes to separate the chloroform layers, which were drawn off through the septum by syringe. The combined organic layers were evaporated to dryness under a nitrogen jet and redissolved in 200 μl of methanol. For HPLC 5 μl to 25 μl aliquots were injected. Smaller reconstitution volumes were permissable where anthracycline levels were low.

Bile. For separation in the reverse phase mode, bile samples (5 μl) could be injected directly, without prior extraction. For

normal phase separation where extraction was necessary, bile samples (200 µl) were extracted five times with equal portions of 9:1 (by volume) ethyl acetate/1-propanol. Very concentrated bile samples were first diluted with saline (1:10 dilution). The combined organic layers were evaporated to dryness under a nitrogen jet and redissolved in 200 µl of methanol, and 5 µl aliquots were injected for HPLC analysis.

Urine. Urine could be injected directly, or, when anthracycline levels were low, the samples could be extracted into ethyl acetate/1-propanol, as for bile.

Quantitation

Several methods were employed to quantitate the chromatographed anthracyclines. Detector response was monitored on a Houston Instrument Co. Omniscribe or Texas Instrument Co. Servo/riter II strip chart recorder and, in some instances, was simultaneously recorded and integrated on a Hewlett-Packard Model 3380A integrator-recorder. For chromatograms obtained with the normal phase column, which produced more tailing, the "cut and weigh" method and peak height x half-peak width gave equally good results. The reverse phase column gave very sharp peaks, and, since the solvent system was a linear gradient, relative peak heights alone gave comparable results to a simultaneous integration by the HP3880A integrator.

Areas or peak heights obtained from the chromatograms of experimental subjects were compared with those of chromatographed standards. Standards consisted of the biological fluid to be examined, obtained from an untreated subject, to which known quantities of ADR, AMNOL, and adriamycinone were added; standards were otherwise handled in the same manner as samples from experimental subjects. Standard calibration curves were linear in the concentration ranges used in this work.

RESULTS AND DISCUSSION

The primary metabolite of ADR is AMNOL, formed by the action of a ubiquitously-occurring aldo-keto reductase (23). Both HPLC systems described here cleanly separate ADR and AMNOL and can be used as a basis for quantifying these two materials. In the Partisil normal phase system, the low polar anthracycline aglycones appear before ADR, generally as a single peak, and can be estimated in terms of adriamycinone equivalents. Polar conjugates, such as O-glucuronides, appear as a broad peak with a rather large k'. In the μ-Bondapak/phenyl reverse phase system, conjugates appear before the AMNOL signal, and adriamycinone elutes after the ADR peak. Glucuronide conjugates may be determined by comparing ADR, AMNOL, and aglycone levels before and after incubation of samples with β-glucuronidase, as previously described (6).

Actual chromatographic separations are illustrated in the accompanying text figures 2-7. Figures 2 and 3 show chromatograms of normal human urine to which pure ADR, AMNOL, and adriamycinone standards have been added, figure 2 as resolved on the μ-Bondapak/phenyl system, and figure 3 as seen with the Partisil PAC 10 system. Figure 4 shows a sample of urine from a patient who received ADR (10 mg/m^2/hr for 8 hours) by continuous infusion into the hepatic artery. Figure 5 shows the separation of ADR, AMNOL, and adriamycinone standards in methanol on μ-Bondapak/phenyl, as determined for retention time analysis, immediately followed by a sample of unprocessed bile collected from a rat bearing a surgically-placed indwelling biliary cannula and given a single dose of ADR, 4 mg/kg, via the left internal jugular vein. Figures 6 and 7 show the analysis of anthracyclines in extracts of human plasma, in one instance by normal phase chromatography and in the other by reverse phase separation.

The limit of detection of ADR and AMNOL for both HPLC systems was, on an absolute mass basis, about 1 ng (1.8 pmoles) per injection; below this level baseline detector noise exceeded peak

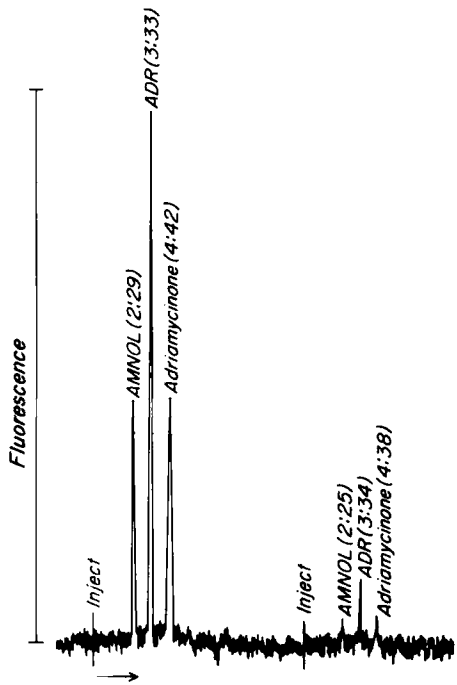


FIGURE 2

Separation on μ-Bondapak/phenyl of ADR, AMNOL, and adriamycinone standards in normal human urine. Each injection was of a 10 μl volume. The stronger signals resulted from concentrations of ADR of 6 μg/ml of whole urine, and of AMNOL and adriamycinone of 3 μg/ml; the weaker signals were obtained from urine containing ADR at 0.50 μg/ml and AMNOL and adriamycinone at 0.25 μg/ml. Retention times (minutes:seconds) are shown on the chromatogram.

height. Fluorescence excitation was done with a deuterium lamp, utilizing the persistent emission of hydrogen at 482 nm, which fortunately comes nearly at the 485 nm excitation maximum of the anthracyclines. Sensitivity could be increased by a factor of 5-10 by exciting at 247 nm, but at this wavelength there may be interfering nonanthracycline fluorescence signals and variable energy loss due to ultraviolet absorbance by a variety of other substances. At 482 nm excitation no interference with the de-

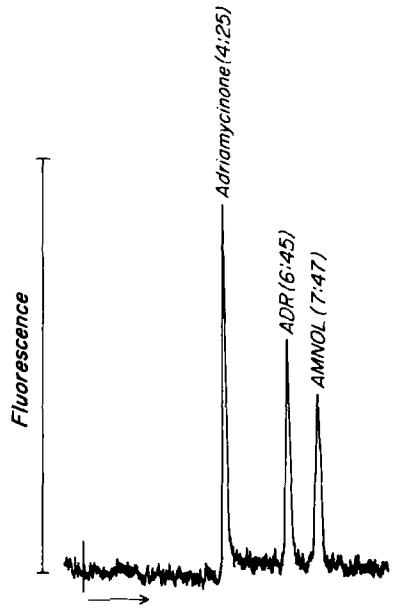


FIGURE 3

Separation on Partisil PAC-10 of ADR, AMNOL, and adriamycinone standards in normal human urine. The signals are derived from a 5 µl injection of urine containing the 3 standards at 5 µg/ml concentration.

tection of ADR, AMNOL, or adriamycinone by native fluorescent materials in plasma, bile or urine was seen, whereas an ultraviolet absorption detector (247 or 254 nm) in series with the fluorometer read numerous peaks. This points out the impossibility of accurately and definitively using ultraviolet absorbance as a means of specifically detecting and quantifying anthracyclines in biological fluids.

By comparison with HPLC injections of ADR and AMNOL standards in methanol, the extraction factor for ADR and AMNOL was determined to be in excess of 70% for plasma, and greater than 86% for bile and urine. It was not necessary to consider these factors, however, since calibration curves were linear over the con-

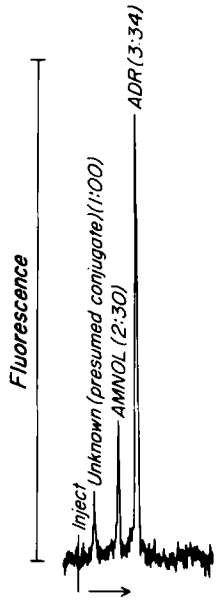


FIGURE 4

Analysis on μ-Bondapak/phenyl of a timed 12 hour urine sample from a patient who received a dose of ADR (10 mg/m^2/hr for 8 hours) by continuous infusion via a hepatic arterial catheter. A 10 μl injection of whole urine revealed a urinary ADR concentration of 4.92 μg/ml, an AMNOL concentration of 1.59 μg/ml, and the presence of an uncharacterized anthracycline fluorescent material (presumed conjugate).

centration range involved (coefficient of correlation > 0.996), thereby taking into account the extractability.

The use in parallel of normal and reverse phase separation systems for ADR and metabolites provides added safety in the identification of fluorescent anthracycline species based upon their chromatographic properties in the two complementary systems. Fluorescent substances which might be tightly bound on one system, thereby escaping detection, should be readily seen on the other system. Comparison of the two methods suggests that the reverse phase system gives cleaner separations and may be performed more

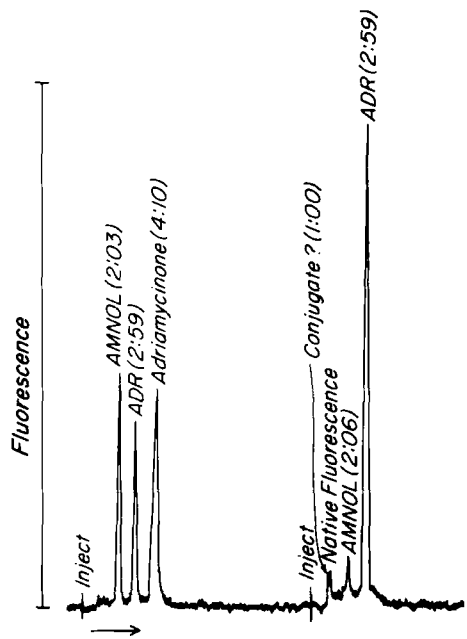


FIGURE 5

Reversed phase (μ-Bondapak/phenyl) separation of ADR, AMNOL, and adriamycinone standard mixture (in methanol), followed by an analysis of 2 μl of whole rat bile from an animal treated with a single intravenous dose of 4 mg/kg of ADR. The bile sample was collected via an indwelling biliary cannula 1.5-2.5 hours after drug administration. ADR was present in the sample at 109 μg/ml concentration, AMNOL at 9.64 μg/ml. The fluorescence signal at 1 min:7 sec was also present in the rat bile prior to drug treatment; a slightly earlier signal appears to be due to an anthracycline conjugate.

rapidly. The longer reequilibration times required for the Partisil PAC 10 column, the somewhat broadened peak shape of AMNOL, and the variability of retention times from day to day point out the major difficulties seen with this system. The limit of detectability of AMNOL (and conjugates, if seen) is somewhat greater than for ADR in the Partisil system, as used here. Modification of the solvent system (for example, use of methanol vs. chloro-

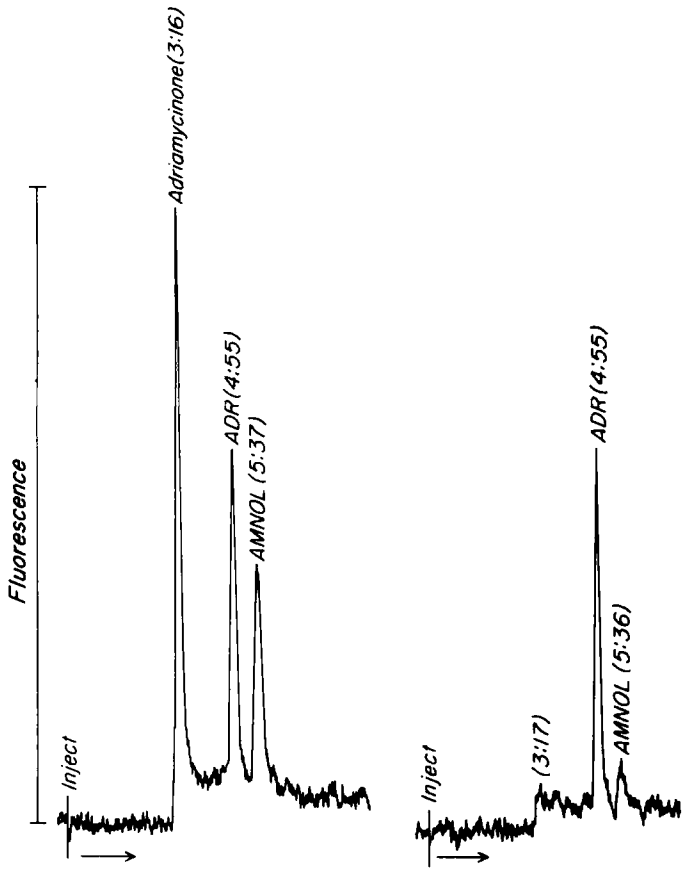


FIGURE 6

Separation on Partisil PAC-10 of a standard 3-component mixture, followed by the analysis of an extract of human plasma from a patient receiving ADR by continuous infusion into a peripheral arm vein. The drug dose was 30 mg/m^2 at an infusion rate of 7.5 mg/m^2/hr for 4 hours. The blood sample was taken 30 minutes after the start of the infusion and was withdrawn via a catheter previously placed in the hepatic artery.

form) can sharpen the AMNOL peak to the point where both ADR and AMNOL are eluted with similar peak shapes during a linear programmed run, but this is accomplished at the expense of adriamycinone,

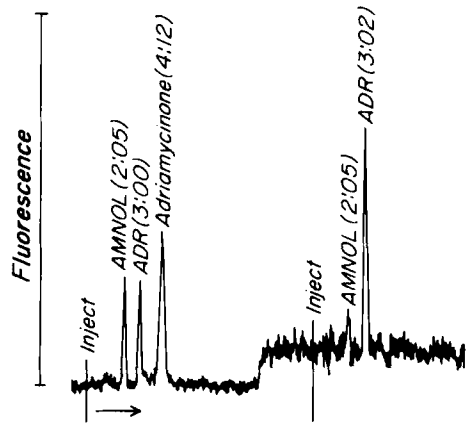


FIGURE 7

The same 3-component mixture and plasma sample as in Figure 6 analyzed on the reversed phase μ-Bondapak/phenyl column.

which is poorly retained and appears as a sharp spike at or close to the void volume. ADR, AMNOL, and adriamycinone have similar peak shapes and similar detector responses on the reverse phase μ-Bondapak/phenyl system, and this is clearly our system of choice for quantifying ADR and metabolites. Nevertheless, we feel that the use of the two complementary analytical systems removes any ambiguity in identification of signals and provides a definitive basis for characterization and quantitation. We recommend their simultaneous use, where possible.

Our recent work with these HPLC systems continues to support our earlier (18) conclusion that plasma, bile, and urine samples of subjects treated with ADR contain little or no free aglycone levels. With modification of extraction procedures, the described methods are now also being applied in our laboratory to the analysis of ADR and metabolites in tissue samples. This methodology should be of general value to other investigators working with ADR experimentally and in the clinic.

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