

## A Simplified Method for Determination of Daunorubicin, Adriamycin, and their Chief Fluorescent Metabolites in Human Plasma by High-Pressure Liquid Chromatography

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**Summary.** A simple method was developed for the routine monitoring of daunorubicin (DR) or adriamycin (ADR) and of their chief fluorescent metabolites in plasma of cancer patients. The plasma samples were treated with ethanol:hydrochloric acid mixture, following which the drug and its metabolites, released to the 40,000 g supernatant, were analyzed by HPLC. A  $\mu$ -bondapak-phenyl column was used and an isocratic mobile phase consisting of acetonitrile in 0.1 M ammonium-formate buffer at pH 4.0. Average recovery of all the tested compounds within the concentration range of 17–3,450 pmol/ml plasma was  $108 \pm 5\%$  (mean  $\pm$  SD).

The method was applied to analyses of plasma samples of several patients treated with DR or ADR. At 3 h after treatment with a DR dose of 45 mg/m<sup>2</sup> or 60 mg/m<sup>2</sup>, daunorubicinol was the major metabolite and its concentrations were 46–270 or 85–305 pmol/ml, respectively; the unchanged drug was present at concentrations of 16–99 or 30–101 pmol/ml, respectively. Deoxydaunorubicinolone and deoxydaunorubicinone were detected at concentrations ranging from 0 to 89 pmoles/ml in the plasma of some patients. Plasma of patients treated with ADR (30 mg/m<sup>2</sup>) contained adriamycinol as the main detectable metabolite, but at 3 h after treatment its concentration was usually lower than that of the unchanged drug ( $22 \pm 9$  vs  $53 \pm 16$  pmol/ml). Traces of 7-deoxyaglycones were detected in some plasma samples.

### Introduction

Daunorubicin (DR) and adriamycin (ADR) are anthracycline antibiotics effective in the treatment of a variety of neoplasms [9, 12, 26]. Their clinical use is limited primarily by cardiac and bone marrow toxicities [9, 12, 19, 23, 27, 28]. The severity of the toxicities is dose-related [9, 27], and can be partly controlled by changing schedules of treatment [5, 11, 28, 29]. Differences among patients in the responsiveness of their leukemia and in toxic side-effects are not well understood, and may be related at least in part to differences in metabolism and distribution of the drugs. Monitoring plasma levels of anthracyclines and their metabolites may yield information that could help our understanding of the reasons for the differences in the responses and may permit a design of treatment schedules suited to individual patients.

Various methods have been used for extraction of DR and ADR from biological samples. Most of them employ an

alcohol-chloroform mixture for extraction of the anthracyclines from plasma into the organic phase, which is then concentrated prior to analysis [1, 6, 17, 20, 21, 28], or reextracted with a small volume of acidic aqueous phase [14]. Other methods include: (i) adsorption of anthracyclines onto a small C<sub>18</sub> column [24] or onto Celite 545 [15], followed by elution with alcohol-chloroform, and concentration; (ii) a several-step extraction procedure starting with a low-temperature acid-alcohol extraction [10]; and (iii) a one-step extraction of adriamycin from plasma, into a sodium hydroxide-acetonitrile mixture which can then be applied directly to a high-pressure liquid chromatograph [22]. It is noteworthy that DR, daunorubicinol, and ADR have been observed to be stable for at least 6 days at about pH 2 [14, 18]. In the present paper we describe a one-step, low-temperature, acid-alcohol extraction from plasma of DR, daunorubicinol, ADR, adriamycinol, and their 7-deoxy and 7-hydroxy aglycones. While this paper was in preparation another method was published [8], describing an acid-mediated extraction of daunorubicin and daunorubicinone.

Mixtures of the anthracyclines, and their metabolites, can be conveniently separated by normal- or reverse-phase high-pressure liquid chromatography [1, 3, 8, 13, 16, 17, 20, 22, 24, 25]. We modified the method of Israel et al. [17] into an isocratic mode, and used it for direct analysis of the acidic extracts. The present method facilitates routine quantitation of daunorubicin, adriamycin, and their chief metabolites, by using a single-step extraction procedure for the anthracyclines, and an isocratic condition for the reverse-phase, high-pressure liquid chromatography.

### Materials and Methods

Daunorubicin (DR, daunomycin) hydrochloride, and adriamycin (ADR, doxorubicin) hydrochloride were purchased from Sigma Chemical Comp., St. Louis, MO, USA. Metabolites of DR and ADR used as reference compounds were generated in our laboratory according to published methods, and are described in detail by Bolanowska and Gessner [7]. The reference compounds were: daunorubicinol (DROL); adriamycinol (ADROL); the 7-deoxyaglycones, namely, 7-deoxydaunorubicinone, 7-deoxydaunorubicinolone, 7-deoxyadriamycinone, and 7-deoxyadriamycinolone; the 7-hydroxyaglycones, namely daunomycinone, daunomycinolone, adriamycinone, and adriamycinolone. We also obtained Farmitalia authentic samples of adriamycinol, and adriamycinone,

through the courtesy of Dr Robert, of Fondation Bergonie, Bordeaux, France. Acetonitrile, glass-distilled, was from Burdick and Jackson Lab., Muskegon, MI, USA. Deionized water was purified using Millipore filters. All other solvents and chemicals were reagent grade.

**High-Pressure Liquid Chromatography.** The HPLC (high-pressure, also called high-performance, liquid chromatography) system consisted of a model 3B HPLC instrument (Perkin-Elmer, Norwalk, Conn., USA) fitted with a pre-packed  $\mu$ -bondapak-phenyl column (3.9 mm  $\times$  30 cm) containing particles 10  $\mu$ m in size (Waters Assoc. Inc., Milford, MA, USA). A solvecon precolumn (Whatman, Clifton, NJ, USA) was installed on-stream between the pump and injector. The Model 7105 syringe loading sample injector with 175  $\mu$ l loop, and column inlet filter (2  $\mu$ m) were manufactured by Rheodyne, Berkley, CA, USA. The mobile phase consisted of acetonitrile at concentration 23%, 27%, or 32%, as further indicated, in 0.1 M ammonium-formate buffer at pH 4.0. It was used isocratically at a flow rate of 2 ml/min. Buffer was filtered through Nylon 66 membrane filter, 0.45  $\mu$ m (PALL Trinity Micro Corp., Cortland, NY, USA) and degassed before use. Detection of the peaks was accomplished with a spectrofluorometer Model 650-10S (Perkin-Elmer, Norwalk, Conn., USA) using excitation at 470 nm and emission at 585 nm. Slit width was set at 10, and the sensitivity range at 10 plus full scale of 'fine' adjustment. Retention time and area of the peaks were recorded by the data station Sigma-10 (Perkin-Elmer, Norwalk, Conn., USA).

**Quantitation.** Standard solutions of the authentic chemicals, ADR, ADROL, adriamycinone, and DR were prepared at concentrations of 20  $\mu$ g/ml in absolute methanol using silanized glass, and were kept at  $-20^{\circ}$  C. Further dilutions

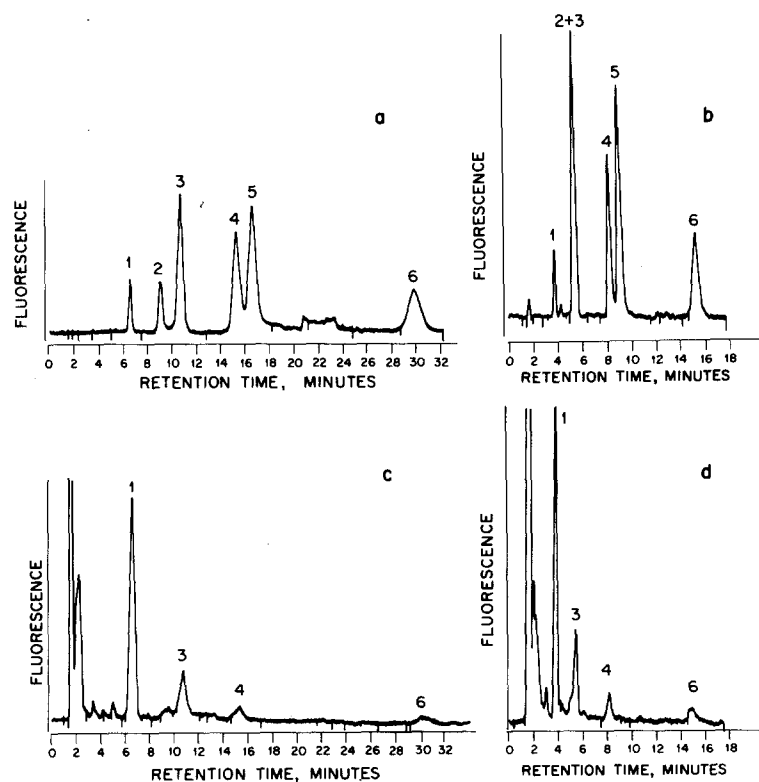
(0.01–1  $\mu$ g/ml) were made with 50% methanol. Calibration curves in the range 0.25–15 ng (0.4–26.0 pmol) were constructed by injecting 5–150  $\mu$ l of the standard solutions onto HPLC column. Peak areas versus concentrations were recorded. The impulse-to-noise ratio of 3 : 1 was achieved at 0.3 ng ADR or DR, and the limit of quantitation was 3 ng/ml plasma. All standard curves were linear ( $r > 0.994$ ) and had intercept 0. The average peak area of 1 pmol was  $0.102 \pm 0.006$  for all the standards. Quantitation of the remaining reference compounds was recorded only in terms of DR, or ADR fluorescent equivalents.

**Patient Plasma.** Plasma of patients undergoing treatment for acute myelocytic leukemia was used in these studies. Heparinized blood from patients treated with daunorubicin or adriamycin, or blank samples withdrawn just before drug injection, were immediately put on ice and kept protected from light. The samples were centrifuged at  $4^{\circ}$  C, at 1,500 g for 15 min. Separated plasma was stored at  $-20^{\circ}$  C. For analysis, a thawed plasma sample was sonicated, and a 0.5 ml aliquot was mixed vigorously with 1 ml 0.6 N hydrochloric acid in absolute ethanol to form a gel. The samples were kept at  $0-4^{\circ}$  C from 3 to 24 h, then centrifuged at 40,000 g for 20 min. Clear supernatant, 50–150  $\mu$ l, was injected onto HPLC column. All manipulations were carried out under subdued lights.

## Results

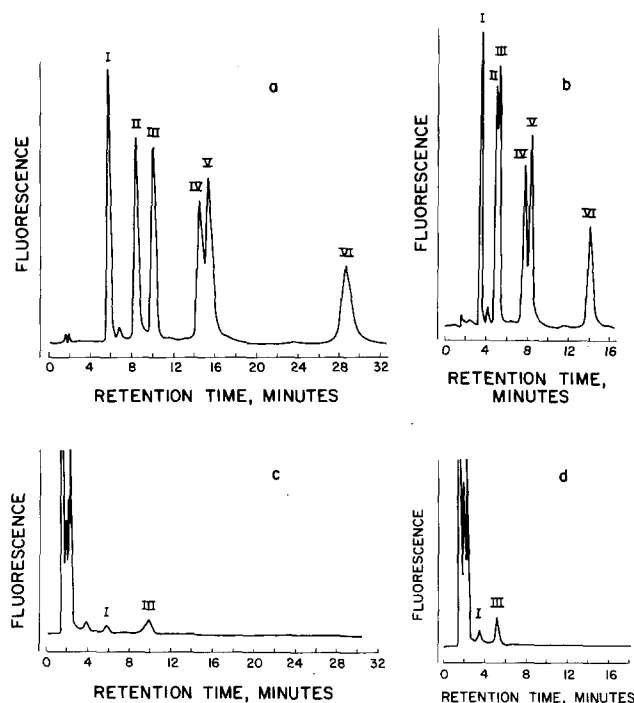
### HPLC Separation of Reference Compounds

DR and its five possible metabolites, also ADR and its five possible metabolites, were separated when 27% or 23% acetonitrile was used in pH 4.0 ammonium formate buffer on a  $\mu$ -Bondapak-phenyl column (see Figs. 1a, 2a and 2b;



**Fig. 1a–d.** HPLC separation of daunorubicin and its metabolites in two mobile phases. Left panel, 27% acetonitrile; right panel, 32% acetonitrile in 0.1 M ammonium-formate buffer, pH 4.0, at flow rate 2 ml/min. Column:  $\mu$ -bondapak-phenyl: a and b show reference compounds; c and d show peaks in plasma of a patient treated with DR. Names of the compounds and assigned peak numbers: 1, daunorubicinol; 2, daunorubicinolone; 3, daunorubicinin; 4, 7-deoxydaunorubicinolone; 5, daunorubicinone; 6, 7-deoxydaunorubicinone

Table 1). The names of the compounds, the assigned peak numbers, and degree of separation are summarized in Table 1. The less polar DR and its metabolites required a somewhat higher acetonitrile concentration than ADR related compounds for comparable mobility.



**Fig. 2a–d.** HPLC separation of adriamycin and its metabolites in two mobile phases. *Left panel*, 23% acetonitrile; *right panel*, 27% acetonitrile in 0.1 M ammonium-formate buffer, pH 4.0, at flow rate 2 ml/min. Column:  $\mu$ -bondapak-phenyl: **a** and **b** show reference compounds; **c** and **d** show peaks in plasma of a patient treated with ADR. Names of the compounds and assigned peak numbers: I, adriamycinol; II, adriamycinolone; III, adriamycin; IV, 7-deoxyadriamycinolone; V, adriamycinone; VI, 7-deoxyadriamycinone

**Table 1.** Separation of daunorubicin and its metabolites and adriamycin and its metabolites, on a reverse-phase HPLC system with different mobile phases<sup>a</sup>

Compound	HPLC peak no.	Capacity factor, $k^b$		
		A	B	C
Daunorubicinol	1	1.28	3.05	—
Daunorubicinolone	2	2.30	4.61	—
Daunorubicin	3	2.30	5.57	—
7-Deoxy-daunorubicinolone	4	4.00	8.32	—
Daunorubicinone	5	4.53	9.13	—
7-Deoxy-daunorubicinone	6	8.30	17.16	—
Adriamycinol	I	0.54	1.14	2.61
Adriamycinolone	II	1.09	2.06	4.26
Adriamycin	III	0.98	2.23	5.30
7-Deoxy-adriamycinolone	IV	1.90	3.77	8.10
Adriamycinone	V	2.16	4.11	8.60
7-Deoxy-adriamycinone	VI	3.85	7.74	16.85

<sup>a</sup> Column  $\mu$ -bondapak-phenyl (3.9 mm  $\times$  30 cm), particle size 10  $\mu$ m: A, 32% acetonitrile; B, 27% acetonitrile; C, 23% acetonitrile, in 0.1 M ammonium-formate buffer pH 4.0 at a flow rate of 2 ml/min

<sup>b</sup>  $k = \frac{R_t(\text{sample}) - R_t(\text{solvent})}{R_t(\text{solvent})}$ , where  $R_t$  = retention time

### Recovery, Calibration, and Stability of Anthracyclines in Plasma

ADR, DR, and their main metabolites, ADROL, DROL, and aglycones, were added to blank plasma in triplicate to give concentrations between 17 and 3,450 pmoles per ml, then the 24-h extraction procedure was applied as described under *Methods*. The samples were analyzed by HPLC; areas of appropriate peaks were recorded and compared with those of the standards prepared in 50% methanol. Percent recoveries of the compounds were tested in repeated experiments ( $n = 3-10$ ) and were as follows (mean  $\pm$  SD): ADR, 9–3,450 pmol/ml (i.e., 5–2,000 ng/ml),  $107 \pm 9$ ; ADROL, 9–3,450 pmol/ml,  $111 \pm 7$ ; adriamycinone, 172 pmol/ml,  $115 \pm 5$ ; 7-deoxyadriamycinolone, 172 pmol/ml,  $103 \pm 3$ ; 7-deoxyadriamycinone, 172 pmol/ml,  $111 \pm 11$ ; DR, 17–3,550 pmol/ml,  $106 \pm 10$ ; DROL 17–3,550 pmol,  $105 \pm 9$ ; daunorubicinone, 89 pmol/ml,  $109 \pm 9$ ; 7-deoxydaunorubicinolone, 178 pmol/ml,  $110 \pm 8$ ; 7-deoxydaunorubicinone, 178 pmol/ml,  $108 \pm 6$ . Average recovery of all the compounds, over the whole range of analyzed concentrations, was  $108\% \pm 5\%$  (mean  $\pm$  SD). The reduced volume of the supernatant compared with that of plasma (before proteins and/or solids were precipitated) could be partly responsible for more than 100% recovery of the added compounds. The high recoveries also attest to the stability of the above compounds in the ethanol–acid extract, over a 24-h period at 0° C.

Calibration plots were also constructed in triplicate for daunorubicin, adriamycin, daunorubicinol, and adriamycinol at concentration 17–1,700 pmol/ml plasma, recovered with a 3-h and a 24-h extraction procedure. Calibration curves were analyzed by regression analysis. In each case a straight-line fit was obtained, with regression coefficient  $r \geq 0.993$  and intercept 0. There was no significant difference between slope values of the calibration plots of the 3-h compared with the 24-h extraction. Average slope values in peak area units per pmole were: 0.098 (ADR), 0.106 (ADROL), 0.111 (DR) and 0.118 (DROL) with SD  $\pm 1.6\%$  of these values.

### Analyses of Patients' Plasma

Plasma withdrawn from cancer patients before treatment with DR or ADR did not in the majority of cases show any peaks interfering with the compounds of interest. Most of the fluorescent natural constituents of plasma were eluted together with the solvent, between 1.5–2.5 min. The samples were analyzed in two systems when a shoulder or an extra peak appeared, to insure correct identification and quantitation of the anthracyclines. Plasma of several patients treated with DR was analyzed in two HPLC systems using mobile phases A and B. It was ascertained that plasma contained daunorubicinol, 7-deoxydaunorubicinolone, and 7-deoxydaunorubicinone, in addition to the parent compound (see Fig. 1c and d). The above metabolites separate well from DR and from each other in the more rapid system A (see Table 1 and Fig. 1), which was used routinely for analysis of patient plasma samples to achieve sharper peaks and reduce time of assay. Daunorubicinone was not detected in plasma, and only a small peak corresponding to daunorubicinolone appeared in some samples (e.g., see Fig. 1c and d).

We analyzed plasma samples of 10 patients treated daily for 3 days with a DR dose of 45 mg/m<sup>2</sup>/day, and of 16 patients treated with 60 mg/m<sup>2</sup>/day. Blood samples were withdrawn just before the treatment and 3 h after each injection, i.e., at 0, 3, 24, 27, 48, 51, and 72 h from the time of the first dose. In all the

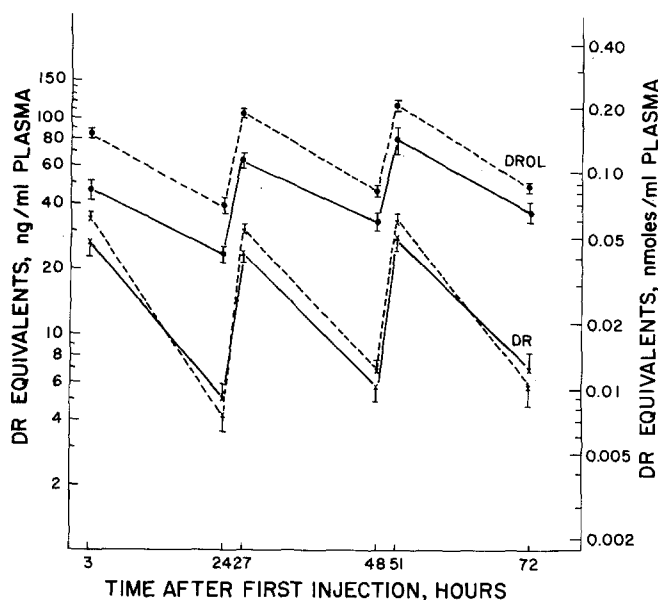


Fig. 3. Concentrations of daunorubicin (DR) and daunorubicinol (DROL) in plasma from patients treated with DR. Solid line, mean value  $\pm$  SE of 10 patients treated daily with DR dose 45 mg/m<sup>2</sup>. Broken line, mean values  $\pm$  SE of 16 patients treated daily with DR dose 60 mg/m<sup>2</sup>. Daunorubicin was injected at 0, 24 and 48 h

samples, the concentration of DROL, the main metabolite of DR, was higher than that of the unchanged drug, as is shown in Fig. 3, which summarizes these results. Concentrations of deoxydaunorubicinolone and deoxydaunorubicinone in plasma of the patients spanned a very wide range, from undetectable level up to 89 pmol/ml plasma.

Plasma samples obtained from several patients at 3 and 24 h after treatment with ADR (30 mg/m<sup>2</sup>) were examined by HPLC, using mobile phases B and C. Pretreatment plasma of patients contained fluorescent constituents that eluted prior to the adriamycinol peak, between 1–4 min in system C. As shown in Fig. 2c and d, adriamycinol was the chief detectable metabolite in patient plasma. At the 3-h time point its mean concentration was  $22 \pm 9$  pmol/ml and at 24 h  $29 \pm 9$  pmol/ml while the respective concentrations of adriamycin were  $53 \pm 16$  and  $16 \pm 6$  pmol/ml ( $n = 18$ ). Traces of 7-deoxyadriamycinolone were detectable in some plasma samples. The 7-hydroxyaglycones, adriamycinolone, and adriamycinone were not detectable in the patient samples that we examined (e.g., see Fig. 2).

## Discussion

The need for a method that would be capable of measuring plasma levels of DR and ADR and their metabolites in cancer patients and could be used routinely by clinical laboratories led us to the studies described here. Extraction of plasma with ethanol–hydrochloric acid for direct use in reverse-phase HPLC analysis offers the advantage of a single-step extraction, with minimal manipulations, and permits analysis of small volumes of plasma. The principle of ethanol : HCl extraction is based on protein precipitation and release of the anthracyclines into the supernatant, which can then be analyzed by HPLC with no additional sample processing. The extraction of anthracyclines from plasma by application of ethanol–HCl overnight has been used previously [2, 4] to determine total fluorescence in such samples. We have determined that this

method extracts, efficiently, and non-destructively, daunorubicin, daunorubicinol, daunorubicinone, adriamycin, adriamycinol, adriamycinone, and deoxy aglycones, when the samples are kept refrigerated throughout the manipulations. The process of release of anthracyclines into the supernatant does not require 24 h, since we demonstrated that good recoveries of the compounds were achieved within 3 h. The procedure calls for a three-fold dilution of plasma with ethanol–HCl mixture, so that small volumes of plasma can be analyzed, since only 50–150  $\mu$ l need to be injected onto HPLC column. We have used routinely 0.5-ml samples for convenience.

The effectiveness of the separation of DR and ADR and their main metabolites by our HPLC system using an isocratic mode is comparable to that achieved by the recently published gradient elution method of Andrews et al. [1]. However, a lack of additional reference compounds that have been used by Andrews et al. [3] precluded a complete comparison of the two chromatographic systems. Nevertheless, considering the limited number of metabolites of DR and ADR found in the clinical samples which we examined, the system described by us offers a practical method for analyses of samples from patients treated with these two drugs, while being simpler and less expensive, because the isocratic mobile-phase system does not require an expensive dual pump chromatograph such as is necessary for a gradient elution.

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