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Quantitation of cell-associated doxorubicin by high-performance liquid chromatography after enzymatic desequestration

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Abstract. A method for measuring cellular concentrations of the anthracycline doxorubicin was developed. The assay involves cell lysis and protein degradation by detergent and proteinase K treatment followed by DNA hydrolysis using DNase I. Prior to high-performance liquid chromatography, samples are deproteinized by the addition of $ZnSO_4$ and methanol. The assay is linear with respect to both the cellular drug content and the number of cells assayed over the ranges tested, and drug recovery is close to 100%. The method has a limit of detection of 50 fmol injected doxorubicin. Within run and between-day coefficients of variation have consistently been found to be in the 5% and 10% range, respectively, in different cell lines exposed to doxorubicin in vitro. The method has been evaluated in analyses of doxorubicin levels in mononuclear blood cells of patients. The assay offers several advantages over commonly used organic extraction techniques and may improve cellular drug monitoring during anthracycline therapy in patients.

Key words: Doxorubicin – HPLC – Cellular concentration

Introduction

The anthracycline doxorubicin is one of the most widely used anticancer agents, with a broad spectrum of activity against a variety of malignancies. The drug is believed to exert its cytotoxic action through a number of mechanisms, including free-radical formation after metabolic activation, intercalation into DNA, induction of DNA breaks, and alteration of cell membranes. The relative contributions of these factors to cell death are at present unknown [9, 30]. However, in vitro studies have demonstrated a relationship between cellular doxorubicin levels and cytotoxicity [29].

Pharmacokinetic investigations in patients treated with doxorubicin have thus far given inconclusive results. Ackland and co-workers [1] have demonstrated a correlation between systemic drug exposure measured as steady-state concentrations and the degree of leukopenia in patients given long-term doxorubicin infusions. In a study of the efficacy of different long-term infusion schedules, Legha et al. [20] demonstrated a positive correlation between peak plasma drug levels and the occurrence of doxorubicin-induced cardiotoxicity. However, no relationship between doxorubicin pharmacokinetics and the therapeutic response has been shown [9], and data from murine models suggest that blood pharmacokinetics may not reflect drug exposure in tumor tissue [6, 7, 8].

In a study of patients given cisplatin or carboplatin for ovarian carcinoma, Reed and co-workers [23] found that the relative content of covalently linked platinum adducts in leukocyte DNA from these patients could predict the therapeutic responsiveness. Similarly, monitoring of cellular doxorubicin concentrations could prove useful for further elucidation of the relationship between anthracycline pharmacokinetics and the therapeutic outcome [10, 17, 27, 28, 32, 34].

Doxorubicin and its biotransformation products are usually analyzed by high-performance liquid chromatographic (HPLC) techniques. Measurement of cellular doxorubicin content has been attempted using a variety of sample-handling approaches. Organic extractions with or without the addition of silver nitrate have been employed by many investigators [4, 5, 7, 11, 12, 14–16, 18, 19, 27, 28, 31–35], whereas others have combined this with trypsin digestion [21]. The methods are laborious and time-consuming and have in most instances been insufficiently evaluated as far as critical parameters such as stability, recovery, and other factors affecting analytical variability are concerned. In the few instances where these factors have been taken into account [17, 28], the assays display a lack of robustness that may limit their usefulness.

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Using two enzymes commonly employed in the manipulation of nucleic acids and a recently published HPLC method [2], we developed a method for the analysis of intracellular doxorubicin that is both straightforward and reproducible. The assay system was evaluated both in vitro and in patients samples.

Materials and methods

Drugs and chemicals. Doxorubicin, doxorubicinol [13(S)-dihydroadriamycin], doxorubicinone, doxorubicinolone (13-dihydroadriamycinone), 7-deoxydoxorubicinone (7-deoxyadriamycinone), and 7-deoxydoxorubicinolone (7-deoxy-13-dihydroadriamycinone) were kind gifts from Dr. A. Suarato (Farmitalia Carlo Erba, Milan, Italy). Proteinase K was obtained from Boehringer Mannheim GmbH (Mannheim, Germany). DNase I and phenylmethylsulfonyl fluoride (PMSF) were supplied by Sigma Chemical Co. (St. Louis, Mo., USA). Formic acid, HPLC-grade acetone, and HPLC-grade isopropanol were obtained from E. Merck (Darmstadt, Germany). All other reagents were of analytical grade. Aqueous reagents and mobile phases were made up in water purified by reversed osmosis followed by polishing with a Milli-Q UF-PLUS system (Millipore Corp., Bedford, Mass., USA).

Apparatus. Chromatographic equipment was produced by Shimadzu Corp. (Tokyo, Japan). The solvent delivery system consisted of a DGU-3A on-line degasser coupled to a LC-9A quarternary gradient pump. The column temperature was maintained using a CT0-6A column oven with an on-line solvent preheater. Samples were injected with a SIL-9A autoinjector maintained at ambient temperature. An RF-551 scanning fluorescence detector was used. Plotting and integration were performed by a Chromatopac C-R6A integrator.

Chromatography. HPLC was performed on a Supelcosil LC18 column (4.6 × 150 mm; particle size, 3 µm; Supelco, Bellefonte, Pa., USA) protected by a 20-mm Supelguard column. The mobile phase consisted of a 0.28 *M* sodium formate buffer (pH 3.55, 24° C): acetone: isopropanol mixture (72.5:25:2.5, by vol.). The mobile phase was delivered at a rate of 1.2 ml/min and the column temperature was maintained at 40° C. The fluorescence detector was operated at an excitation wavelength of 500 nm and an emission wavelength of 580 nm. In all, 100 µl of sample was injected. The method is described in detail elsewhere [2].

Standard solutions. Standards of doxorubicin and the five metabolites were made up in methanol, aliquoted and stored at –70° C until their use. Concentrations in the standards were verified by measurement of the absorption at 495 nm with a Shimadzu UV-1201 spectrophotometer.

Cell culture and patients samples. The myeloid leukemia cell lines HL-60 and KG1a, the breast-cancer cell line T-47D, and the breast-cancer cell strain KFLb1 were maintained in logarithmic phase by dilution in recommended growth media. The T-47D and KFLb1 cells form adherent layers in tissue-culture flasks, whereas the other cell lines tested grow in suspension. The cells were diluted to starting densities of 5×10^5 cells/ml prior to doxorubicin exposure, incubated at 37° C in an atmosphere of 5% CO_2 in air for 24 h, and subsequently harvested, counted (electronic particle counter model ZM, Coulter Electronics Ltd., Luton, UK), and washed in ice-cold phosphate-buffered saline (PBS).

Cell nuclei were isolated as described elsewhere [25]. Cells were briefly shaken in a buffer containing 0.32 *M* sucrose, 10 m*M* TRIS-HCl, 5 m*M* $MgCl_2$, and 1% Triton X-100 (pH 7.6) and were then spun (12,000 *g* for 60 s), after which the supernatant was discarded. This procedure was repeated until the supernatant was clear (i.e., 1–2 times), and the nuclei were counted. The entire procedure was carried out on ice.

Blood from patients undergoing doxorubicin therapy was obtained in citrated vacuum tubes (Becton Dickinson, Rutherford, N.J., USA) and the plasma was separated by centrifugation. Mononuclear blood cells were isolated by sodium metrizoate-Ficoll density-gradient centrifugation (Lymphoprep; Nycomed, Oslo, Norway), counted, and washed.

Sample preparation. The entire procedure was carried out in single 1.5-ml microcentrifuge tubes. Cells were resuspended in 400 µl ice-cold PBS, and 10 µl each of Triton X-100 (5%) and proteinase K (10 mg/ml) was added. After brief mixing, the sample was incubated for 1 h at 65° C in a water bath. Then 5 µl PMSF (10 m*M* in isopropanol) was added and the sample was left on the bench for 10 min prior to the addition of 10 µl $MgCl_2$ (0.4 *M*) and 20 µl DNase I (1 mg/ml). After brief centrifugation (to ensure that droplets did not form on the inside of tube), the sample was incubated in a water bath at 37° C for 30 min.

Deproteinization of samples was undertaken as described for plasma samples [2]. To 450 µl of sample was added 450 µl of methanol and 45 µl of $ZnSO_4$ (400 mg/ml). After mixing, the samples were centrifuged at 15,000 *g* for 5 min in a Biofuge 13 microcentrifuge (Heraeus Sepatech, Osterode, Germany). Then 800 µl of the supernatant was transferred to borosilicate glass autosampler vials (Chromacol Ltd., London, UK) prior to HPLC analysis.

Results

The sample preparation procedure is illustrated in Fig. 1. The method is based on cell lysis and degradation of cellular proteins with a combination of Triton X-100 and the endopeptidase proteinase K. Following the addition of a serine protease inhibitor, DNA is hydrolyzed by treatment with DNase I in the presence of divalent cations.

Apparent intracellular doxorubicin concentrations in drug-exposed KG1a cells as a function of the incubation time with DNase I are shown in Fig. 2. Stable drug recovery was attained after 30 min. A standard curve was constructed by adding known concentrations of doxorubicin to untreated KG1a cells, which were subsequently subjected to the sample handling procedure (Fig. 3A). Likewise, linearity was determined by assaying different numbers (10^5 –10^7) of drug-exposed cells (Fig. 3B). As is evident from Fig. 3, there was a linear response with respect to both the added drug and the amount of cells analyzed over the range tested.

Drug recovery was assessed by adding doxorubicin, doxorubicinol, and the other four doxorubicin metabolites to KG1a and HL-60 cells prior to sample preparation. Absolute recovery was in the 85%–105% range for all compounds after addition to both cell lines (Table 1). In the absence of DNase treatment, the recovery of doxorubicin, doxorubicinol, and, to a lesser extent, the other four compounds, was dramatically reduced (Table 1, bottom). Without the addition of proteinase K during sample preparation, doxorubicin recovery was reduced by approximately 90%.

The impact of DNase concentration and incubation time on absolute drug recovery was determined by varying the incubation conditions. When doxorubicin-spiked cell pellets were incubated with one-tenth of the DNase concentrations, recoveries were 70% ($n = 2$) and 82% ± 3% (mean ± SD, $n = 4$) after 30 and 60 min of incubation, respectively. As is evident from Table 2, doubling of both

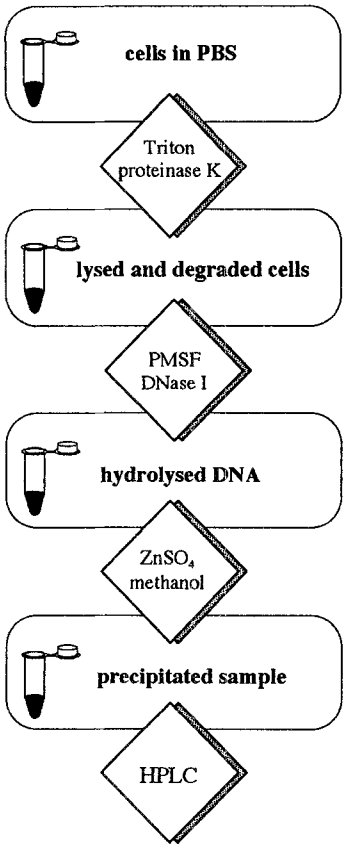

Fig. 1. Schematic presentation of the sample handling procedure for analyzing cellular doxorubicin levels

the DNase concentration and the incubation time had no effect on the recovery of doxorubicin and its metabolites. Likewise, the use of other nonionic detergents such as Nonidet P-40 (NP-40; 0.1%) or polysorbate 20 (Tween-20; 0.1%) or increasing the Triton X-100 concentration to 1% did not affect the drug recovery. Within-run and between-day assay variability was determined in several cell lines after exposure to 100 n*M* doxorubicin for 24 h. The coefficients of variation are given in Table 3.

We analyzed cells directly after harvesting, following storage at –70° C, and after partial extraction (including the addition of PMSF but not DNase) and storage at –70° C. We observed no discernible difference in the analytical results obtained after the different procedures (data not

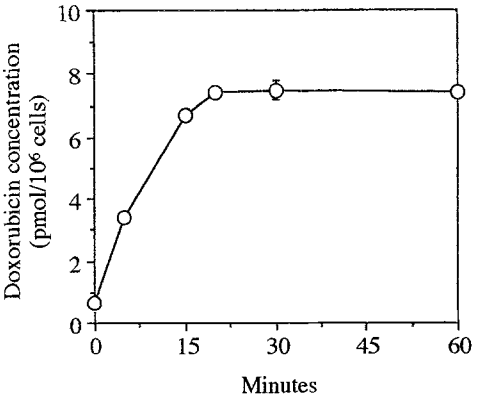

Fig. 2. Apparent cellular doxorubicin concentrations in KG1a myeloid leukemia cells as a function of incubation time with DNase I. Cells were cultured as described in Materials and methods, exposed to 100 n*M* doxorubicin for 24 h, harvested in aliquots of 5×10^6 cells, and analyzed for doxorubicin content. Error bars (30 min) represent 1 SD ($n = 4$)

Table 1. Recovery of doxorubicin and five metabolies as determined at one concentration

Compound	Cell line, number of cells	Drug concentration (pmol/10^6 cells)	Recovery	
			%	CV
Doxorubicin	KG1a,	20	89	5.2
Doxorubicinol	5×10^6	20	93	4.9
Doxorubicinone		4	97	1.8
Doxorubicinolone		4	89	3.1
7-Deoxydoxorubicinone		4	86	4.7
7-Deoxydoxorubicinolone		4	94	3.3
Doxorubicin	HL-60,	20	102	2.8
Doxorubicinol	5×10^6	20	92	3.9
Doxorubicinone		4	100	4.1
Doxorubicinolone		4	98	3.8
7-Deoxydoxorubicinone		4	98	4.0
7-Deoxydoxorubicinolone		4	105	6.9
Doxorubicin	KG1a[a],	20	10	
Doxorubicinol	5×10^6	20	22	
Doxorubicinone		4	56	
Doxorubicinolone		4	65	
7-Deoxydoxorubicinone		4	48	
7-Deoxydoxorubicinolone		4	63	

Each data point, with the exception of recoveries from KG1a cells in the absence of DNase, is based on 5 different analyses. CV, Coefficient of variation

[a] No DNase I was added during sample preparation

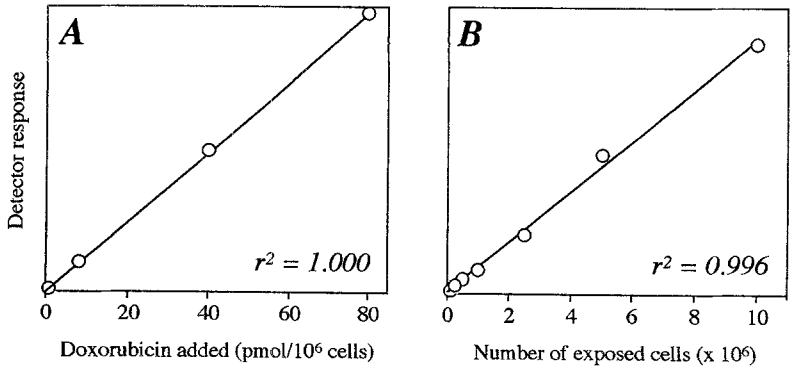

Fig. 3A, B. Assay linearity as a function of **A** doxorubicin concentration and **B** the total number of cells assayed. The myeloid cell lines KG1a and HL-60 were cultured as described, harvested, and analyzed. The figures depict representative experiments. **A** Mean values for 3 replicates (CV < 5%) after spiking of untreated KG1a cells (5×10^6) with different concentrations of doxorubicin. **B** Results of analyzing different numbers of HL-60 cells ($10^5 - 10^7$) after exposure to 100 n*M* doxorubicin for 24 h in vitro. Correlation coefficients of regression lines are given

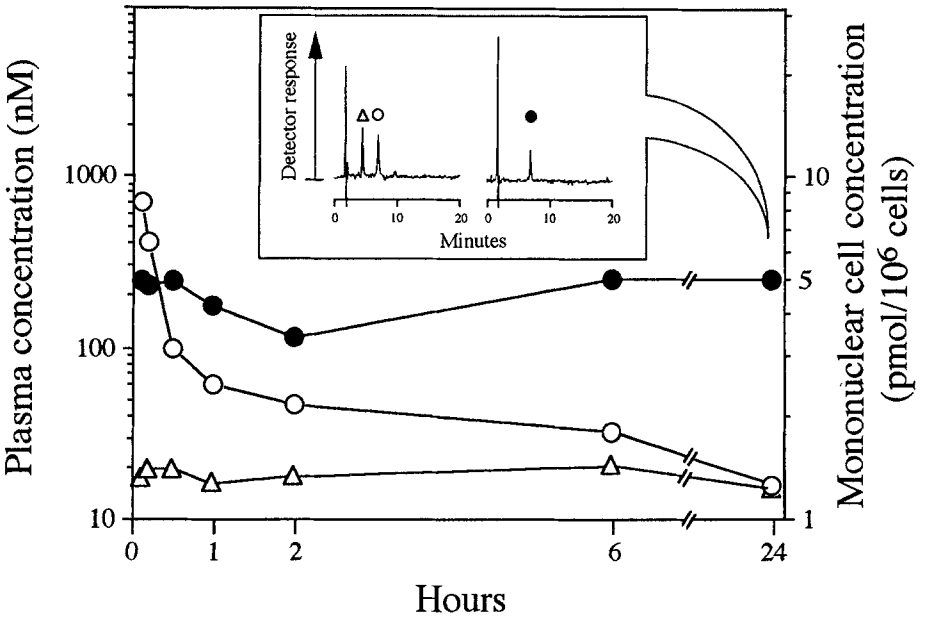

Fig. 4. Concentrations of plasma (*open symbols*) and mononuclear cell (*filled symbols*) doxorubicin (*circles*) and doxorubicinol (*triangles*) in a lymphoma patient given 50 mg/m^2 doxorubicin as a short-term intravenous infusion. *Insert:* Chromatograms of the plasma (*left*) and mononuclear cell (*right*) samples at 24 h

Table 2. Recovery of doxorubicin and five metabolites as determined at one concentration

Compound	Cell line, number of cells	Drug concentration (pmol/10^6 cells)	Recovery (%)
Experiment 1:			
Doxorubicin	HL-60,	10	100
Doxorubicinol	5 ×10^6	10	101
Doxorubicinone		2	105
Doxorubicinolone		2	96
7-Deoxydoxorubicinone		2	91
7-Deoxydoxorubicinolone		2	106
Experiment 2:			
Doxorubicin	HL-60,	10	103
Doxorubicinol	5 ×10^6	10	102
Doxorubicinone		2	107
Doxorubicinolone		2	94
7-Deoxydoxorubicinone		2	92
7-Deoxydoxorubicinolone		2	104

During experiment 1, cell samples were incubated with the given concentration of DNase for 30 min. In experiment 2, cells were incubated twice as long in double the concentration of the enzyme. Each data point is based on 1–3 separate experiments

Table 3. Assay variability in different cell lines incubated with 100 n*M* doxorubicin for 24 h

Cell line	*n*	Mean concentration (pmol/10^6 cells)	CV (%)
Within-day variability:			
KG1a	4	7.5	4.1
KG1a	4	7.1	5.7
KG1a	2	7.8	
KG1a nuclei	4	6.7	4.7
HL-60	4	19.8	2.0
HL-60	5	24.8	4.9
HL-60	4	24.0	7.1
HL-60 nuclei	4	17.7	2.7
T-47D	2	90	
KFLb1	4	55	2.1
Between-day variability:			
KG1a	5		9.4
HL-60	4		12.4

shown). The stability of samples was also investigated after the end of sample preparation. Storage of samples at room temperature in a dark environment for 24 h or refrigeration for up to 2 weeks had no effect on the analytical variability (data not shown).

Figure 4 depicts the plasma and mononuclear cell pharmacokinetics of doxorubicin in a lymphoma patient given 50 mg/m^2 doxorubicin as a bolus injection. The plasma disposition-curve kinetics were described by a two-compartmental model with a rapid distribution phase ($t_{1/2\alpha}$, 0.1 h) followed by a protracted elimination phase ($t_{1/2\beta}$, 14 h). In marked contrast, drug levels in mononuclear cells remained virtually unchanged during the first 24 h (Fig. 4).

Discussion

Intracellular anthracycline levels have been monitored in numerous studies over the last two decades. However, quantitation of these drugs in cells and tissues has encountered methodological difficulties, which to a large extent are due to a combination of these drugs' high affinity to cellular constituents and their chemical instability [6]. Thus, both fundamental and clinical studies of the cellular pharmacology of anthracyclines have been hampered by problems associated with assay precision, sensitivity, and specificity.

In 1973, Schwartz [26] reported a method for measuring doxorubicin and daunorubicin in tissues. The technique involved extraction of drug by a combination of $AgNO_3$ and isoamyl alcohol followed by fluorometry of the solvent. Drug recoveries were quantitative and the assay was linear over the range of concentrations tested [26]. The

major disadvantages of this method are its lack of sensitivity and specificity. Although the assay has been modified for HPLC-based detection [11], current techniques mainly rely on the organic extraction of drug [5, 10, 13, 15, 17, 18, 19, 22, 24, 27–29, 31–35]. A variety of organic solvents such as chloroform, ethylacetate, trichloroacetate, acetonitrile, and 1–5 carbon alcohols have been used for single [3, 5, 11, 13, 17, 18, 19, 22, 24, 32–35] or repeated [10, 15, 16, 27, 28, 31] extractions in volumes of up to 16 ml/sample [15]. Some investigators have analyzed the organic solvent without any evaporation or reconstitution step [3, 11, 12, 22, 24, 32]. In the few instances where the methods have been carefully evaluated, both low extraction volumes and high cell numbers have been found to reduce analytical recoveries considerably [17, 28].

Herein we report a novel enzymatic sample-preparation procedure for the quantification of cellular doxorubicin. The method relies on well-proven enzymatic techniques to dissociate proteins and hydrolyze cellular DNA. The use of proteinase K not only ensures degradation of native proteins but also prevents doxorubicin biotransformation by cellular enzymes present in the sample [25]. The proteinase K is then inactivated by the addition of PMSF. The toxic properties of this substance could limit the applicability of the current method in clinical settings. On the other hand, PMSF is inactivated in aqueous solutions in a matter of hours and therefore does not constitute a permanent environmental hazard [25]. Although we did not evaluate them, the recently developed protease inhibitors (4-amidinophenyl)-methanesulfonyl fluoride (APMSF; Boehringer Mannheim, Mannheim, Germany) and 4-(2-aminoethyl)-benzenesulfonyl fluoride (Pefabloc SC; Pentapharm Ltd., Basel, Switzerland) reportedly offer similar inhibitory properties with greatly reduced toxicity. The addition of DNase I in the presence of Mg^{2+} hydrolyzes DNA in a random fashion to a complex mixture of mono- and oligonucleotides [25], thus dissociating the doxorubicin intercalated in structurally intact DNA. After the enzymatic release of drug, the reaction mixture is deproteinized by the sequential addition of $ZnSO_4$ and methanol and analyzed by a sensitive, isocratic HPLC technique [2].

Detergent was used to facilitate cell lysis. Sodium dodecyl sulfate, which is most efficient in this respect, could not be included in our system due to interferences during HPLC separation, presumably due to the introduction of ion-pairing effects. A comparison of different concentrations of Triton X-100 and other nonionic detergents (NP-40, Tween-20) showed no significant difference. Sample preparation was optimized by systematic variation of the concentration of different reagents. The method is critically dependent on the sequential addition of both proteinase K and DNase I in the sense that omission of either results in an approximately 90% reduction in analytical recovery. Experiments (Figs. 2, 3; Table 2) gave no indication that enzymes and inhibitor were present in anything but excessive quantities.

Attempts to extract doxorubicin from cells solely by precipitating proteins with $ZnSO_4$ and methanol have resulted in mean recoveries of about 13% as compared with the present method (data not shown). We have also tried extracting the drug with combinations of $AgNO_3$ and organic solvents prior to HPLC [11]. This approach was abandoned due to major baseline fluctuations during quantitation, which are not surprising in view of the photochemical properties of $AgNO_3$ in the detector light beam. With the exception of a method that employs trypsin digestion in concert with organic extraction [21], the present assay is, at least to our knowledge, the first enzymatic sample-handling method for the analysis of cellular anthracycline levels.

The present method offers good analytical linearity (Fig. 3A) and has a limit of detection (S:N ratio, 3:1) of 50 fmol injected [2]. The assay allows the quantification of intracellular doxorubicin irrespective of the number of cells analyzed. We evaluated the sample-preparation procedure with initial cell numbers in the 10^5-10^7 range (Fig. 3B) and with several different cell types without any indication that the assay performance is dependent on the absolute amount of starting material. Analytical recoveries of both doxorubicin and five of its metabolites were quantitative (Table 1), and the assay reproducibility was adequate in the cell lines tested (Table 3). One intriguing aspect of the recovery studies is that in HL-60 cells not subjected to DNase treatment, recoveries of doxorubicin and its metabolites varied in the 10%–65% range, with the lowest recoveries being observed for the parent compound and the metabolite doxorubicinol (Table 1, bottom). Of the six doxorubicin compounds tested, these moieties are the only two that display cytotoxic properties [30]. One could speculate as to whether the low in vitro recovery observed in the absence of DNase treatment reflects an interdependence between DNA intercalation and bioactivity.

We evaluated the assay system in patient samples, and an example of the elimination kinetics observed after intravenous treatment with doxorubicin in a single patient is given in Fig. 4. In this subject, doxorubicin was cleared from the plasma with distribution and elimination half-lives of 0.1 and 14 h, respectively, whereas a constant amount of doxorubicin was sequestered in mononuclear leukocytes over the period studied (Fig. 4). This finding is in agreement with the results of previous investigations [30, 32, 34].

In summary, the present assay offers quantitative recovery of cellular doxorubicin with good linearity and reproducibility. The method may be of use for elucidating the relationship between intracellular anthracycline levels and the treatment outcome during pharmacotherapy with drugs of this class.

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