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Improved high-performance liquid chromatography assay of doxorubicin: Detection of circulating aglycones in human plasma and comparison with thin-layer chromatography

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Summary. *We compared doxorubicin and metabolite pharmacokinetic data obtained from thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) assay of plasma samples from six patients who had been treated with doxorubicin. Duplicate 1-ml samples were extracted with chloroform : isopropanol (1 : 1) and assayed using a sensitive HPLC system incorporating a dual pump gradient with tetrahydrofuran as the mobile phase and fluorescence detection. Duplicate 1-ml samples from the same specimens were assayed using a modification of a previously described TLC assay. Areas under the curve for doxorubicin by HPLC (3.36 ± 2.30 μM · h) and TLC (4.16 ± 2.50 μM · h) were not significantly different (P = 0.5). Terminal half-life of doxorubicin by HPLC (28.0 ± 6.98 h) and TLC (23.2 ± 7.8) (P = 0.29) and the calculated total-body clearances by HPLC (0.55 ± 0.29 l/min) and TLC (0.45 ± 0.23) (P = 0.55) were not significantly different. Areas under the curve for doxorubicinol by HPLC (2.75 ± 1.4 μM · h) and TLC (2.53 ± 7.1 μM · h) (P = 0.73) showed no significant differences. HPLC detected a mixed 7-deoxydoxorubicinol aglycone–doxorubicin aglycone peak, 7-deoxydoxorubicin aglycone, and two nonpolar, unidentified metabolites. TLC detected the following aglycone metabolites: doxorubicin aglycone, doxorubicinol aglycone, 7-deoxydoxorubicinol aglycone, an unidentified polar metabolite, and several unidentified nonpolar metabolites. From these data we conclude that HPLC and TLC detect concentrations of doxorubicin and doxorubicinol from human plasma equally well to concentrations of 7.0 nM (4 pmol injected doxorubicin). Aglycones do circulate in human plasma at concentrations above the detection limits of both assays. Doxorubicinol aglycone, which is detected by TLC but not by HPLC, may be formed from artifactual breakdown of doxorubicinol during TLC development. Unidentified nonpolar compounds seen on HPLC and TLC may represent further doxorubicin metabolism than previously described.*

Introduction

Many groups have described accurate rapid, high-performance liquid chromatographic (HPLC) assays for doxorubicin [1, 2, 4, 8, 9, 11, 12, 14–16], but none has succeeded in routinely detecting metabolites other than parent compound (doxorubicin) and the major reduction metabolite (doxorubicinol) extracted from human plasma. This has fueled speculation that aglycone metabolites do not circulate in humans [10]; yet, thin-layer chromatography (TLC) data suggest the presence of multiple aglycone metabolites [6]. No confirmation of the presence of these metabolites in human plasma has been provided by other assay techniques.

The toxicologic and therapeutic importance of aglycone metabolites remains unknown. In part, this is because commonly used assays are unable to detect the low concentrations of aglycones in human plasma. In this communication we report our modification of a previously published HPLC assay for doxorubicin [11] and its metabolites. The modifications allow detection of circulating aglycone metabolites. Furthermore, we have compared our modified HPLC assay with the standard TLC assay in use in other laboratories [6].

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Materials and methods

Materials. Doxorubicin hydrochloride was purchased as commercial material from Adria Laboratories and was purified for use by chromatography on 0.25 mm silica gel 60 thin-layer chromatography (TLC) (EM Reagents). Tetrahydrofuran (THF) was obtained from Fisher Laboratories (HPLC grade) and was degassed prior to use. Ammonium formate (Fisher certified) buffer, 0.1% (w/v) was made fresh daily with distilled water adjusted to pH 4.0 with formic acid (Fisher certified). The buffer was subsequently filtered (Millipore, Milford, Mass, 0.45 μm) and degassed before use. For chemical extraction, chloroform (Fisher certified), 2-propanol (Fisher certified), and ammonium sulfate (Fisher certified) were used.

Synthesis of metabolites. The following metabolites were synthesized according to the published procedures of Takakashi and Bachur [17]: doxorubicinol, doxorubicin aglycone, doxorubicinol aglycone, 7-deoxydoxorubicin aglycone, and 7-deoxydoxorubicinol aglycone. Purity of all standards was confirmed by a single peak on HPLC at published retention times [1]. No other peaks in the HPLC trace suggested at least 99% purity of the single peak metabolites. Identity of the standards was confirmed by mass spectroscopy.

Extraction. Our extraction and assay procedures were modified from those published by Andrews et al. [1]. Briefly, for this procedure an extraction of 1 ml plasma in 2 ml 1 : 1 (v/v) chloroform : isopropanol was used. The extraction yielded approximately 1.7 ml organic phase, which was evaporated to dryness under a nitrogen jet and was reconstituted in 100 μl 7 : 3 (v/v) methanol : tetrahydrofuran HPLC injection.

HPLC analysis. For the analysis, 60 μl of this solution was injected into the HPLC. A dual-pump HPLC (Waters

Associates, Millipore Corp, Milford, Mass) was used with a fluorescence spectrophotometer and Data Module (Waters Associates, Millipore Corp., Milford, Mass) for metabolite detection and peak quantitation. The specimen was assayed on a 10-μm pore size μ-Bondapak phenyl HPLC 30 cm column (Waters Associates, Millipore Corp, Milford, Mass) using a 10-min linear gradient and a 5-min washout. The gradient consisted of a starting concentration of 15% tetrahydrofuran in 16 m*M* formate buffer to a final concentration of 50% tetrahydrofuran. We modified the published procedure as follows: (a) the amount of daunorubicin internal standard added was decreased to 0.05 nmol from 0.5 nmol; (b) the Waters Associates (Millipore Corp., Milford Mass) HPLC system was modified by the addition of a WISP 71OB automatic injector (Waters Associates, Millipore Corp., Milford, Mass) and replacement of the solvent programmer with a Computerized Systems Controller (Waters Associates, Millipore Corp., Milford, Mass) for coordination of the automatic injector, two pumps, and the Data Module (Waters Associate, Millipore Corp., Milford, Mass) (curve integrator); and (c) the fluorescence detector was replaced by a Perkin-Elmer Model 650-40 fluorescence spectrophotometer fitted with an 18-μl quartz rectangular micro-flow cell accessory (Perkin-Elmer Corp., Norwalk, Conn). The excitation wavelength was 467 nm and the emission detection wavelength was 550 nm. To protect against photolytic degradation, all assays and procedures were protected from direct exposure from fluorescent light. The HPLC was completely protected from light by the use of steel tubing.

Within- and between-day variability of the HPLC assays was evaluated by extraction of variable concentrations of doxorubicin from plasma (0.007–0.1 nmol/ml) against a daunorubicin internal standard (0.05 nmol/ml). Duplicate extractions were injected on successive days. The average slope (± SD) of the plots was 16.48 ± 2.03, and the average correlation coefficient was 0.988 ± 0.016. Pooled human plasma samples spiked with doxorubicin, doxorubicinol, and aglycone metabolites were stored overnight in a freezer and then defrosted, extracted, and assayed by HPLC. No evidence of artifactual breakdown of doxorubicin, doxorubicinol, or aglycones was noted following HPLC assay.

Quantitation. A standard curve from each individual patient's pretreatment plasma was prepared. The ratio of known doxorubicin peak area to daunorubicin peak area (internal standard) was calculated. Following linear regression analysis, concentrations of metabolites and doxorubicin from plasma were calculated from their ratio to the internal standard (daunorubicin).

Our quantitations of metabolites were based upon assumptions previously published [1]. These assumptions suggested that ε, the molar extinction coefficient at the excitation wavelength, and Φ, the quantum efficiency, are equal for doxorubicin and its known metabolites when F, a compound's fluorescence, is defined as $F = KI\,\Phi\,(\varepsilon\,bc)$ [11], where I is the incident radiant power, b the path length, c the concentration, and K the proportionality constant. We also assumed, as in the previous work [1], that the extraction efficiencies of doxorubicin and the metabolites were identical with that of daunorubicin. Although data have been generated to dispute these assumptions [1], we are unable to develop separate standard curves in plasma for each metabolite because of the limited amount of plasma available from each patient. Therefore, we report metabolite concentrations as 'doxorubicin fluorescent equivalents' (μ*M*).

TLC analysis. Thin-layer chromatographic assay was performed with modifications of the published technique of Benjamin et al [6]. Specimens were extracted in duplicate with 1 : 1 chloroform : isopropanol. The dried residue was redissolved in 100 μl chloroform : methanol (1 : 1, v/v). The entire volume of the extracted material was layered on a 0.25-mm silica gel 60 TLC plate (EM reagents). Duplicate specimens were layered on separate plates. Each TLC plate was co-chromatographed with doxorubicin and metabolite standards. Plates were consecutively developed in ethyl acetate; chloroform : methanol : acetic acid : water (80 : 20 : 14 : 6, v/v/v/v); and chloroform : methanol : water (100 : 15 : 15, v/v/v). The plates were dried and fluorescent metabolites, parent compound, and co-chromatographed standards were identified by a 254-nm ultraviolet light source. Bands between doxorubicin and doxorubicinol were considered polar metabolites and labeled P. Bands with greater metabolite mobilities than doxorubicin aglycone that did not co-chromatograph with known standards were labeled MNP or mostly nonpolar metabolites.

Quantitation was obtained by scraping an identifiable band and eluting the fluorescence with 75% methanol: 0.6 *N* HCl. Corresponding areas of the silica gel plate containing no sample were similarly processed for use as blanks. Total drug fluorescence in plasma, a measure of doxorubicin and its fluorescent metabolites, was determined in duplicate after extraction with 75% methanol: 0.6 *N* HCl (acid alcohol) as previously published [5]. The plasma concentration of each fluorescent species was obtained by determining its percent concentration on the chromatogram and multiplying this percentage by the total drug fluorescence obtained after acid alcohol extraction. All fluorescence determinations were performed in a 4-ml quartz cuvette on a Perkin-Elmer Model 650-40 fluorescence spectrophotometer (Perkin-Elmer Corp, Norwalk, Conn) fitted with a standard size cuvette holder. The excitation wavelength was 467 nm and emission was detected at 585 nm.

Data analysis. Data were fit by means of the nonlinear curve-fitting program MLAB [13] to the equation $C(t) = Ae^{-\alpha t} + Be^{-\beta t} + Ce^{-\gamma t}$, where A, B, and C are constants and α, β, and γ are the apparent first-order elimination rate constants. A 1/(concentration)^2 weighting function was used to fit the curve. The area under the curve (AUC) was calculated by the log-trapezoidal method with extrapolation to infinity. Total body clearance (Cl_{TB}) was calculated using the equation: Cl_{TB} = Dose/AUC. Pharmacokinetic data from TLC and HPLC assays were compared by unpaired Student's *t*-tests.

Patients. Six patients undergoing treatment with doxorubicin 60 mg/m^2 as IV bolus injections consented to the study. After admission to the Vanderbilt University Clinical Research Center, informed consent, approved by the Vanderbilt University Committee for the Protection of Human Subjects, was obtained. Other than necessary narcotic analgesics in two patients and metoclopramide as necessary for nausea and vomiting, no other drugs were administered to these patients during or after their treatment. Samples of 12 ml blood were obtained in heparinized tubes at 0.25, 1, 2, 4, 8, 12, 24, 36, 48, and 72 h after doxorubicin administration. After immediate

centrifugation at 4° C the plasma was withdrawn and immediately frozen at −20° C for assay. All assays were performed within 4 weeks of specimen storage. Because of the variability among individuals in preferential plasma protein binding of doxorubicin over daunorubicin, all standard curves were produced in individual patients' plasma [1].

Results

Assay system

Chloroform-isopropanol extraction efficiency for both HPLC and TLC assays was determined by comparison of HPLC areas of doxorubicin extracted from pooled human plasma and

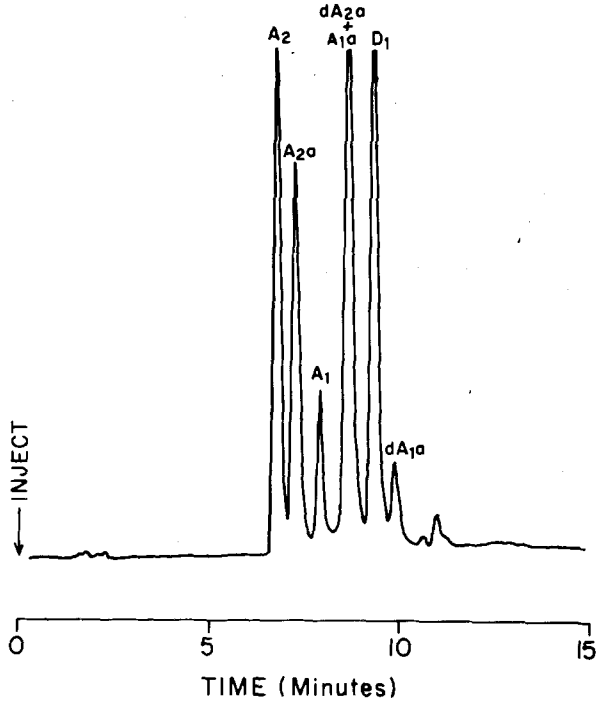


Fig. 1. HPLC separation of pooled doxorubicin metabolites synthesized at Vanderbilt University

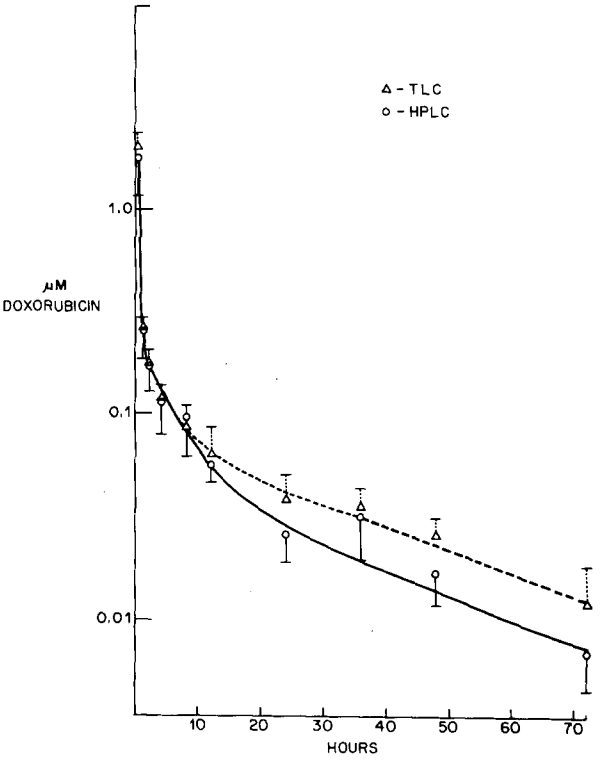


Fig. 3. Doxorubicin plasma disappearance curves – HPLC and TLC assays; data from six patients receiving doxorubicin alone, 60 mg/m^2 IV over 5 min

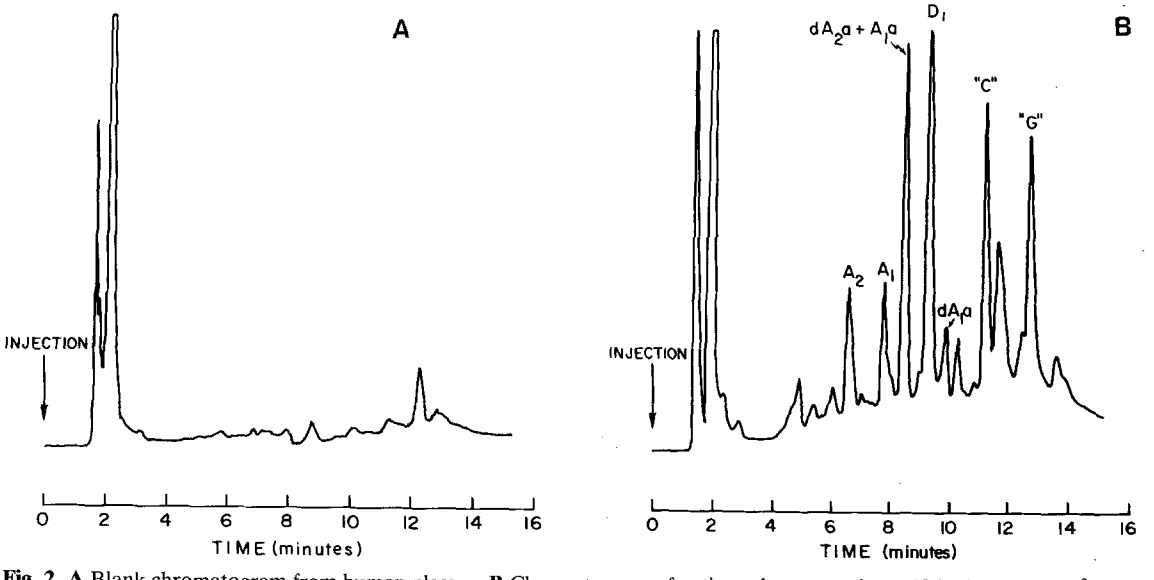


Fig. 2. A Blank chromatogram from human plasma; **B** Chromatogram of patient plasma specimen 12 h after 60 mg/m^2 doxorubicin given IV over 5 min. Labeled peaks: A_1, doxorubicin; A_2, doxorubicinol; dA_1a, 7-deoxydoxorubicin aglycone; dA_2a, 7-deoxydoxorubicinol aglycone; A_1a, doxorubicin aglycone; *C*, suspected metabolite C; *G*, suspected metabolite G

Table 1. Identification of doxorubicin and its metabolites, with comparison of capacity factors from previous studies [1] and current work

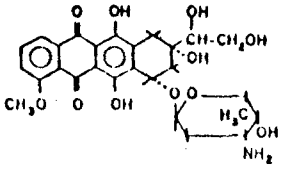
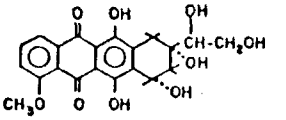
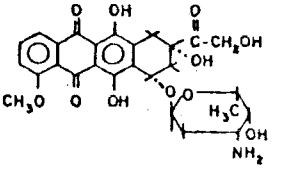
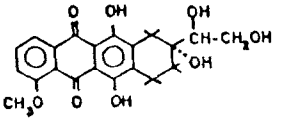
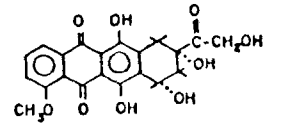
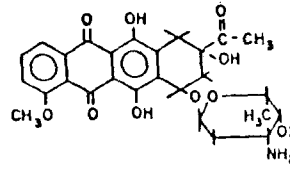
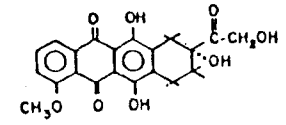
Structure	Abbreviation	Compound	k' (1)	k'
	A_2	Doxorubicinol	5.00	5.25
	A_2a	Doxorubicinol aglycone	6.00	5.74
	A_1	Doxorubicin	6.63	6.43
	dA_2a	7-Deoxydoxorubicinol aglycone	7.25	7.19
	A_1a	Doxorubicin aglycone	7.25	7.19
	D_1	Daunorubicin	7.88	7.90
	dA_1a	7-Deoxydoxorubicin aglycone	8.38	8.48

Table 2. Pharmacokinetic data: TLC vs HPLC assay of doxorubicin and doxorubicinol

	HPLC	TLC	P
Doxorubicin			
$C \times t$ ($\mu M \cdot$ h)	3.36 ± 2.30	4.16 ± 2.5	0.5
$t_{1/2}$ (h)	28.0 ± 6.98	23.2 ± 7.80	0.29
C_p° (μM)	2.0 ± 2.59	2.33 ± 1.37	0.80
Cl_{TB} (l/min/m^2)	0.55 ± 0.29	0.45 ± 0.23	0.55
Doxorubicinol			
$C \times t$ ($\mu M \cdot$ h)	2.75 ± 1.4	2.53 ± 7.1	0.73

Table 3. Comparison of doxorubicin pharmacokinetic data in individual patients

Patient no.	AUC ($\mu M \cdot$ h)		$t_{1/2}$ (h)	
	HPLC	TLC	HPLC	TLC
1	2.45	4.95	24.8	34.7
2	2.89	2.10	30.6	19.8
3	7.63	6.66	20.4	30.1
4	2.08	1.93	34.7	14.4
5	3.98	7.39	36.5	22.7
6	1.10	1.98	20.8	17.3

injected in methanol standard at concentrations from 0.01 μM to 1.0 μM. Extraction efficiencies were variable in the range of concentrations assayed. Efficiencies ranged from 50% to 90% but did not increase directly with concentration. The addition of the internal standard for HPLC analysis enabled us to overcome the problems of intraduplicate and interspecimen extraction variability. Previous data [1] yielded similar extraction efficiencies for doxorubicin metabolites. For TLC analysis, the relative percentages of the metabolites were not affected by variable extraction efficiencies. The acid alcohol

Figure 1 demonstrates HPLC separation of doxorubicin and the synthesized metabolites from our laboratory. The calculated capacity factors (k′) for our metabolites were similar to those obtained in previous work (Table 1) [1]. Occasionally interfering fluorescent substances in pretreatment plasma required us to subtract background fluorescent peaks from known peaks. Rarely, we were unable to assess individual metabolites due to background fluorescence. Representative chromatograms of blank human plasma extracts and treated human plasma extracts are shown in Figs 2A and B, respectively. The detection limit of our system, defined as a signal-to-noise ratio of 3 : 1 was 0.1 pmol (0.054 ng) of doxorubicin injected from a standard methanol solution. The detection limit of doxorubicin extracted from 1 ml plasma was 4 pmol injected on the column.

Comparison of TLC to HPLC pharmacokinetic data

Plasma disappearance curves of doxorubicin and of doxorubicinol are similar (Fig. 3 and 4). Pharmacokinetic data comparing HPLC and TLC assay techniques for doxorubicin and doxorubicinol are shown in Table 2. Table 3 compares individual patients' pharmacokinetic data for doxorubicin. While there was no significant difference between the pharmacokinetics derived from the two techniques for the assay of doxorubicin or doxorubicinol, individual studies suggested important differences between the assays.

TLC and HPLC detected different metabolites. The TLC assay suggested that higher concentrations of the aglycone metabolites of doxorubicin and doxorubicinol (Fig. 5A and 5B) exist in human plasma. HPLC assay suggested that at least two previously unidentified nonpolar metabolites (Fig. 6) circulate and can be routinely detected. AUC data for all metabolites are listed in Table 4.

The synthesis of known aglycone metabolites and their co-chromatography with plasma specimens on each TLC plate have led to improved accuracy of TLC analysis. We found routine co-chromatography of standards necessary because of variations in fluorescent band mobility of the same metabolite between duplicate patient plasma specimens developed in parallel TLC plates in identical solvent solutions. Co-chro-

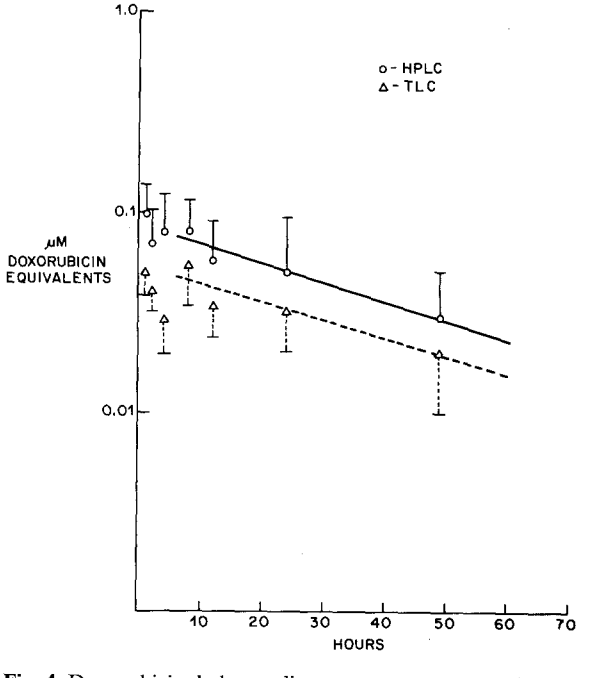
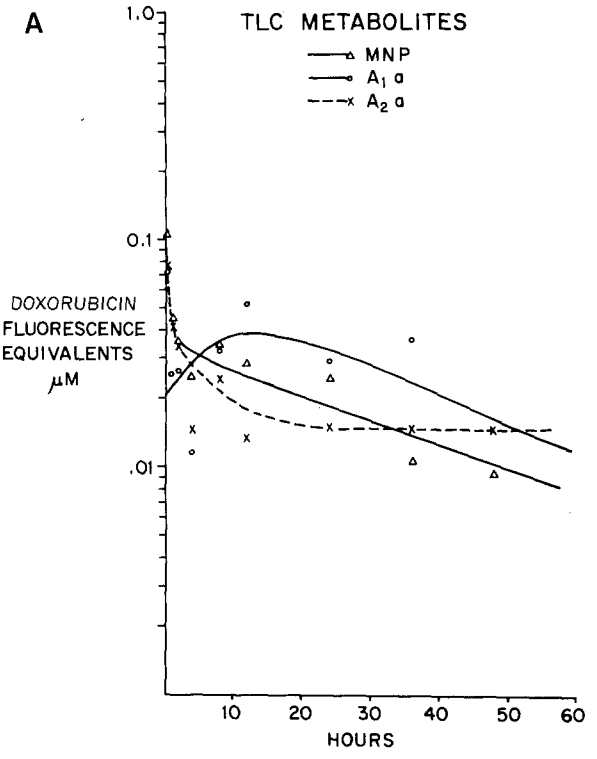


Fig. 4. Doxorubicinol plasma disappearance curves – HPLC and TLC assays; data from six patients receiving doxorubicin alone, 60 mg/m^2 IV over 5 min



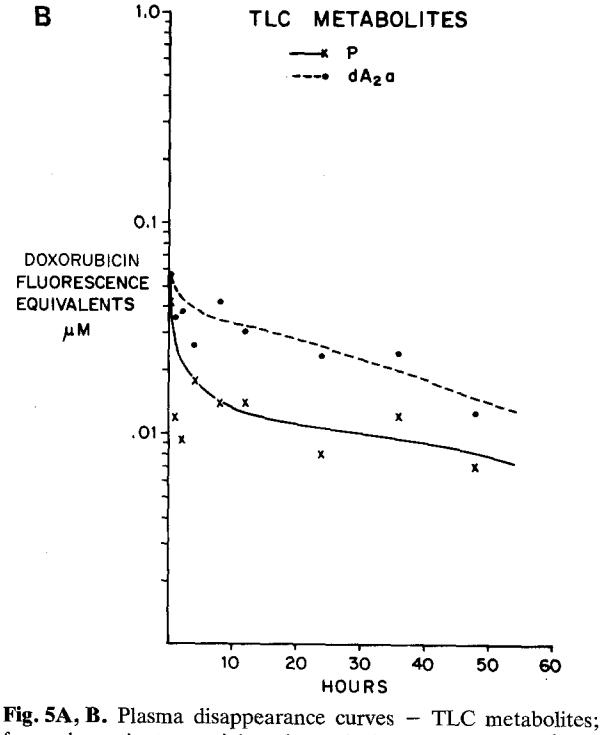


Fig. 5A, B. Plasma disappearance curves – TLC metabolites; data from six patients receiving doxorubicin alone, 60 mg/m^2 IV over 5 min

Table 4. Doxorubicin metabolites: $C \times t$ ($\mu M \cdot h$) of TLC- and HPLC-detected metabolites

Metabolite	*P*	A_2a	MNP	dA_2a	A_1a	dA_1a	C	G
TLC	0.978 ± 0.913	1.66 ± 1.26	1.73 ± 0.93	1.72 ± 1.50	2.08 ± 3.36	ND	ND	ND
HPLC	ND	ND	ND	dA_2a/A_1a = combined	3.13 ± 4.86	0.425 ± 0.313	2.14 ± 3.20	0.885 ± 0.661

Figures give means ± SD

[a] A_2a, doxorubicinol aglycone; dA_2a, 7-deoxydoxorubicinol aglycone; MNP, mostly nonpolar fluorescent metabolite; A_a, doxorubicin aglycone; dA_1a, 7-deoxydoxorubicin aglycone; C and G, unidentified nonpolar metabolites, P, polar fluorescent metabolite

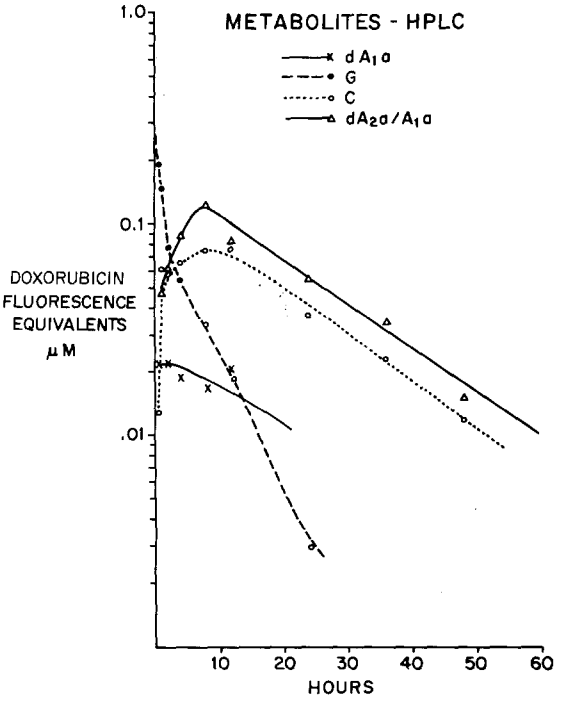


Fig. 6. Plasma disappearance curves – HPLC metabolites; data from six patients receiving doxorubicin alone, 60 mg/m² IV over 5 min

matography with known standards resolved this problem. Intraduplicate variability of calculated metabolite concentrations from parallel plates was rarely greater than 10%.

Discussion

Our modification of a previously published HPLC technique [1] by the addition of an improved fluorescence spectrophotometer incorporating a low-volume flow cell demonstrates the importance of detector technology for the assay of anthracyclines. The improvement in detector technology and the use of continuous 1.6 mm OD, 0.23 mm ID tubing from the column outlet to the detector have improved the sensitivity of our HPLC anthracycline assay system approximately seven-fold. This improvement in sensitivity has allowed us to detect both known and suspected metabolites on a routine basis from human plasma extractions.

For the assay of doxorubicin and doxorubicinol, we find that both TLC and HPLC are accurate and reproducible to human plasma concentrations of 7 n*M*. The plasma disappearance curves and derived pharmacokinetic data confirm this contention, yet we found differences among individual patient studies. We cannot find any single reason to explain the discrepancies between the techniques for individual patient specimens. We suspect that multiple factors, particularly the labor-intensive silica scraping, variable extraction of drug from the silica by the methanol–0.6 *N* HCl, and variable cleavage of daunosamine sugars by the acidic developing solutions for the TLC assay, may explain some discrepancies. Variability in protein binding, lipid residue anthracycline binding, and binding to glass surfaces may account for further differences between the techniques. In spite of these difficulties, final pharmacokinetic modeling did not reveal statistically important differences between the techniques for the assay of doxorubicin.

Assay of doxorubicin metabolites also revealed important differences between the techniques. HPLC detected peaks in a region where doxorubicin metabolites were previously unidentified. These peaks were consistent at 11.20 min and 12.72 min from patient to patient and exhibited exponential declines. We suspect the peaks we have identified are nonpolar fluorescent metabolites, and we have arbitrarily labeled them as metabolites C (11.20 min peak) and G (12.72 min peak). These peaks may represent further evidence of doxorubicin's complex metabolism, and may represent the AAL fraction observed in the published TLC data of Benjamin et al. [6]. The importance and structure of these peaks remain unknown.

TLC assays detected higher concentrations of doxorubicinol aglycone and doxorubicin aglycone. While doxorubicinol pharmacokinetic data were not statistically higher in HPLC-assayed specimens than in TLC data, we noticed a trend for higher doxorubicinol concentrations in HPLC assays. No doxorubicinol aglycone was detected by HPLC, whereas doxorubicinol aglycone was a prominent metabolite when assayed by TLC. We suspect that acid hydrolysis, as suggested by Glode [10], does occur during TLC assay and affects the resolution of doxorubicinol. This may explain why doxorubicinol aglycone was found in the TLC assay but not in the HPLC assay.

In summary, our data suggest that despite individual differences, the HPLC or TLC assay can be used for doxorubicin and doxorubicinol. Metabolites obtained with the two techniques differ, probably reflecting differences in the pH and buffer systems used. The HPLC assay detects nonpolar metabolites which need to be identified in the future. TLC assay detects aglycone metabolites, but it suffers from the possibility of some degree of acetic acid-induced hydrolysis of doxorubicinol from the developing solutions. HPLC is unable to separate the twin peaks of doxorubicin aglycone and 7-deoxydoxorubicinol aglycone. Concentrations of these two metabolites must be estimated from TLC at present.

In our view, HPLC is the preferred technique for anthracycline assay. Large numbers of specimens may be assayed by this automated technique and the data obtained are equal in quality to those yielded by TLC. Future improvements

in column technology may allow resolution of the joint aglycone peaks and further improvements in the detection of nonpolar metabolites. HPLC permits collection of unknown metabolite peaks, which will allow their identification in the future. We plan to use HPLC technology for our continuing work with anthracyclines.

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