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Note

High-performance liquid chromatographic determination of doxorubicin and its metabolites in plasma and tissue

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Doxorubicin (DOX, adriamycin) is an anthracycline antibiotic used in the treatment of a variety of human neoplastic disease [1]. Many high-performance liquid chromatographic (HPLC) procedures have been reported for the determination of this drug and its main metabolite, the 13-dihydro derivative (DOL) [2–19]. In attempts to reproduce the analytical procedures of previously reported methods [8,15], we encountered a number of problems, some of which have been previously noted by other investigators: (a) irreproducible quantitation of standards due to adherence of DOX to glass and plastic surfaces [20]; (b) complex extraction procedures [13]; (c) the presence of insoluble material in extracts of plasma and lung tissue; (d) extraction of fluorescing substances that co-elute with DOX and its metabolites; (e) irreproducibility of reported recoveries [13]; (f) a retention time for DOX too short to allow proper quantitation; and (g) a retention time for the internal standard too long to allow rapid processing of samples. Because of these problems, we proceeded to develop both an extraction and an HPLC procedure for DOX and its metabolites. In this report, we describe a one-step extraction of DOX and its metabolites from plasma and tissue, followed by direct injection of the extract for analysis by reversed-phase, gradient HPLC.

EXPERIMENTAL

Materials

DOX and daunorubicin aglycone were supplied by the National Cancer Institute (Washington, DC, U.S.A.). DOL was prepared by a reported procedure [21]. Resolution of compounds of interest was achieved with a 10-μm LiChrosorb RP-

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18 column (250 mm × 4.6 mm I.D.) from Keystone Scientific (State College, PA, U.S.A.). A guard column containing the same packing material preceded the analytical column. All organic solvents were glass-distilled, HPLC grade and were purchased from Curtis-Matheson Scientific (Marietta, GA, U.S.A.). Deionized, distilled Milli Q water was used for all work performed.

Apparatus

For HPLC analysis, a Waters Assoc. ALC 202 high-performance liquid chromatograph equipped with a Rheodyne injector and a Perkin-Elmer LS-4 fluorescence spectrometer was used. Column effluent was monitored at an excitation wavelength of 475 nm and an emission wavelength of 580 nm at a sensitivity of 1.0. Slit widths were 10 nm for excitation and 20 nm for emission. Glassware used for homogenization of lung tissue was silanized with dichlorodimethylsilane in toluene (5:95, v/v) and washed with HPLC-grade methanol.

Preparation of extracts

Plasma extracts were prepared by mixing plasma with four volumes of methanol containing daunorubicin aglycone as an internal standard. Precipitated protein was removed by centrifugation, and 100-μl portions of the clear supernatants were injected directly for HPLC analysis.

Tissue extracts were prepared by adding first one volume of methanol followed by two volumes of Tris buffer (1 *M*, pH 8.5). The mixtures were homogenized, and the homogenates were allowed to stand on ice for 15 min before seven volumes of acetonitrile containing the internal standard were added. The mixtures were vortexed and allowed to stand at room temperature for 15 min before the precipitated protein was removed by centrifugation. Portions (100 μl) of the clear supernatants were injected for HPLC analysis.

HPLC procedure

HPLC analyses were performed with a 10-min linear gradient from 25 m*M* $NH_4H_2PO_4$/0.03 *M* phosphoric acid–acetonitrile (85:15, v/v) to 25 m*M* $NH_4H_2PO_4$/0.03 *M* phosphoric acid–acetonitrile (1:1, v/v). The flow-rate was 1.5 ml/min. Between analyses, the column was equilibrated at initial conditions for 15 min between analyses.

RESULTS AND DISCUSSION

In preparation for in vivo studies, extracts of plasma and lung tissue, to which DOX had been added, were prepared and analyzed by HPLC [8,15]. Because of the problems we encountered in the application of these methods to our samples, we developed both extraction and HPLC procedures for DOX and its metabolites.

With solutions of DOX in methanol, a standard curve was generated for a range of concentrations on column from 1.5 to 155 pmol. This curve was characterized by a correlation coefficient of 0.99, and R.S.D. of 3% and an accuracy of 6%. The minimum amount reproducibly detected and quantitated was 1.5 pmol. In three experiments involving extraction of DOX (345 pmol/ml) from plasma, recov-

eries were 92, 93 and 98%. When varying amounts of these extracts were analyzed, the integrator response was linear. For concentrations of 172–860 pmol/ml, these results were confirmed, with recoveries of 90–95%. The lowest concentration reproducibly quantitated was 17 pmol/ml. In all experiments, the range of recovery for the internal standard was 97–102%.

Duplicate samples of DOX in plasma (17, 34, 172 and 1720 pmol/ml) were held on ice for 2 h, then stored at −10°C for one week. The thawed samples were extracted and analyzed as described above. The results showed that DOX was stable under these conditions.

The procedure for extraction of DOX from plasma, which was simpler and more efficient than previously reported procedures [2,15], was not adequate for lung tissue. In order to develop an appropriate procedure, we considered reports [15,18] that extraction of DOX from an aqueous medium with an organic solvent was pH-dependent, with the optimum range of pH 8.0–8.6. One of the reports [18] emphasized that self-association of DOX in the aqueous phase substantially influenced the distribution ratio. When duplicate samples of DOX in lung tissue (1.5, 5, 50 and 250 nmol/g) were extracted with methanol–Tris (pH 8.5)–acetonitrile, the average recovery was 80%, with a range of 60–98%. The range of recovery for the internal standard was 77–86%. These values are in agreement with those reported by Shinozawa et al. [12] and Robert [13]. Robert also reported that he was unable to reproduce reported extraction recoveries and that recoveries were concentration-dependent unless large volumes of extraction solvent were used [13].

These procedures were applied to samples from B6C3FI mice. Doses of DOX (17 μmol/kg) were injected intravenously into the tail veins of groups of four B6C3F1 mice; blood and lung tissue were collected at 5, 30 and 120 min. Blood samples were collected in heparinized tubes and centrifuged to collect plasma. Lung tissue was frozen on dry ice immediately after excision. All samples were stored frozen overnight before they were extracted for analysis, as described above. DOX, DOL, a major unidentified metabolite, and several minor unidentified metabolites were separated from plasma (Fig. 1). The concentration of DOX decreased rapidly between 5 and 120 min (Table I). The concentrations of DOL, which has fluorescence properties similar to DOX [17], were lower but followed the same pattern. The concentration of the major unidentified metabolite, which eluted between DOX and the internal standard and which was quantitated as having fluorescence equivalent to DOX, appeared to increase with time. In lung tissue, the concentration of DOX was highest at 30 min after dosing and lowest at 5 min (Table I). In this tissue, no metabolites of DOX were evident (Fig. 1). In a previous study [22], only trace amounts of metabolites were noted in the lungs of hamsters dosed with DOX (5 mg/kg).

In agreement with previous reports [15], our data indicate that DOL is the major metabolite of DOX. The minor metabolites that we observed in plasma may be aglycone derivatives, which have also been identified [23].

In these procedures, it is necessary that solutions of DOX in methanol be used for standard curves and for evaluation of extraction procedures [15]. If aqueous solutions are used, even with polypropylene containers or silanized glassware, as

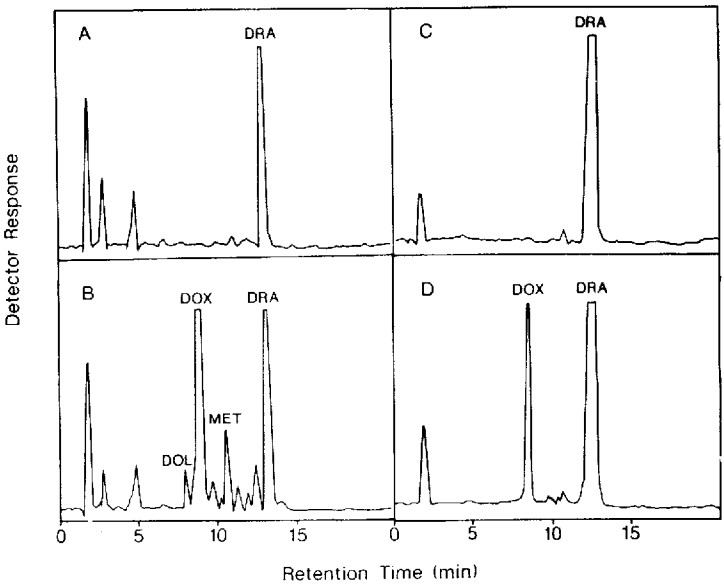

Fig. 1. Separation of DOX and its metabolites from fluorescing components of plasma and lung tissue. (A) Extract of plasma with added internal standard (DRA); (B) extract of plasma of a mouse 5 min after dosing with DOX (10 mg/kg); (C) extract of lung tissue with added DRA; (D) extract of lung tissue from a mouse 30 min after dosing with DOX (10 mg/kg). MET = unidentified metabolite of DOX.

TABLE I

LEVELS OF DOX AND ITS METABOLITES IN PLASMA AND LUNG TISSUE OF MICE AFTER A DOSE OF 17 μmol/kg

Recovery of the internal standard for this experiment was 97–100% for plasma and 77–86% for lung tissue. Values were corrected accordingly.

Time (min)	Concentration (mean ± S.D., $n=4$)			
	Plasma (pmol/ml)			Lung (pmol/g)
	DOX	DOL*	Unidentified metabolite	DOX
5	2115 ± 313	108 ± 49	93 ± 24	2468 ± 415
30	208 ± 20	37 ± 11	73 ± 24	5901 ± 1640
120	162 ± 20	16 ± 3	126 ± 86	3163 ± 1207

*The identification of this component as DOL is based on retention time only.

recommended in previous reports [20,24], reproducible amounts of the compound cannot be transferred from the solutions. Also, it should be noted that, as the analytical HPLC column ages, it is necessary to adjust the acetonitrile content of the buffers in order to maintain reproducible retention times for the components of interest.

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REFERENCES

1 S.K. Carter, J. Natl. Cancer Inst., 55 (1975) 1265.
2 N. Erb, R. Erttmann and G. Landbeck, Cancer Chemother. Pharmacol., 17 (1986) 53.
3 G. Vasanthakumar and N.K. Ahmed, Biochem. Pharmacol., 35 (1986) 1185.
4 A.N. Kotake, N.J. Vogelzang, R.A. Larson and N. Choporis, J. Chromatogr., 337 (1985) 194.
5 H. Iguchi, H. Tone, T. Ishikura, T. Takevchi and H. Umezawa, Cancer Chemother. Pharmacol., 15 (1985) 132.
6 D.E. Brenner, S. Galloway, J. Cooper, R. Moore and K.R. Hande, Cancer Chemother. Pharmacol., 14 (1985) 139.
7 H. Ichiba, M. Morishita, T. Yajima, Y. Kawatsu, M. Kawamura, H. Komatsu and T. Ikegami, Chem. Pharm. Bull., 33 (1985) 3868.
8 M. Broggini, C. Italia, T. Colombo, L. Marmonti and M.G. Donelli, Cancer Treat. Rep., 68 (1984) 739.
9 J. Cummings, J.F.B. Stuart and K.C. Calman, J. Chromatogr., 311 (1984) 125.
10 A. Aszalos, J. Liq. Chromatogr., 7 (1984) 69.
11 W. Bolanowska, T. Gissner and H. Preipler, Cancer Chemother. Pharmacol., 10 (1983) 187.
12 S. Shinozawa, Y. Mimaki, Y. Araki and T. Oda, J. Chromatogr., 196 (1980) 463.
13 J. Robert, J. Liq. Chromatogr., 3 (1980) 1562.
14 R. Baurain, D.D. Campeneere and A. Trouet, Cancer Chemother. Pharmacol., 2 (1979) 11.
15 R.N. Pierce and P.I. Jatlow, J. Chromatogr., 164 (1979) 471.
16 P.M. Wilkinson, M. Israel, W.J. Pegg and E. Frei III, Cancer Chemother. Pharmacol., 2 (1979) 121.
17 S. Eksborg, H. Ehrsson and I. Andersson, J. Chromatogr., 164 (1979) 479.
18 S. Eksborg, J. Chromatogr., 149 (1978) 225.
19 S. Eksborg, J. Pharm. Sci., 66 (1977) 782.
20 A.G. Bosanquet, Cancer Chemother. Pharmacol., 17 (1986) 1.
21 T.H. Smith, A.N.Fujiwara and D.W. Henry, J. Med. Chem., 22 (1979) 40.
22 N.R. Bachur, M.J. Egorin and R.C. Hildebrand, Biochem. Med., 8 (1973) 352.
23 C.E. Riggs, R.S. Benjamin, A.A. Serpick and N.R. Bachur, Clin. Pharmacol Ther., 22 (1977) 234.
24 E. Tomlinson and L. Malspies, J. Pharm. Sci., 71 (1982) 1121.