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Analysis of 4-Demethoxydaunorubicin and Metabolites in Plasma and Urine

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A high-performance liquid chromatographic procedure, including sample pretreatment, is presented for the analysis of 4-demethoxydaunorubicin, the C_{13} hydroxy metabolite 4-demethoxydaunorubicinol and four possible aglycone metabolites, 4-demethoxydaunorubicinone, 4-demethoxydaunorubicinolone, 4-demethoxy-7-deoxydaunorubicinone and 4-demethoxy-7-deoxydaunorubicinolone, in plasma and urine samples. Doxorubicin has been utilized as the internal standard of the assay. The sample pretreatment involves liquid–liquid extraction of the buffered (pH 9) biological matrix with chloroform–1-propanol (4 : 1, v/v). Separation of the compounds has been achieved by using a Lichrosorb RP-8 (5 μm) column and by isocratic elution with a mobile phase composed of acetonitrile–phosphate buffer, pH 2.4 (70 : 30, w/w). The flow rate is 1.5 ml/min. Fluorescence detection with excitation at 460 nm and monitoring at 540 nm was applied. The detection limit is 1 ng/ml (using 1.0 ml samples). The applicability of the assay has been demonstrated in a pharmacokinetic study with two rabbits. In plasma, 4-demethoxydaunorubicinol and 4-demethoxydaunorubicinolone were observed as metabolites, whereas 4-demethoxydaunorubicinol was the main metabolite present in urine. Fitting of the plasma concentration *versus* time curves showed a three-compartment model to give a better description of the plasma concentration–time curves than a two-compartment model.

Keywords——4-demethoxydaunorubicin; anthracycline; HPLC; pharmacokinetics

Introduction

The anthracycline antibiotic doxorubicin (Dx) is unquestionably one of the most powerful antineoplastic agents presently in use. However, Dx produces acute haematological toxicities as well as treatment-limiting damage to the myocardium at total cumulative doses exceeding 550 mg/m^2, which impedes continuation of Dx chemotherapies.[1–3] The adverse drug effects have prompted many research programs with the objective of overcoming these undesirable side effects, without attenuation of antitumour efficacy.[4] Studies have been conducted on co-administration of radical scavengers such as vitamin E,[5] modification of drug dosage regimens,[6] encapsulation of Dx in liposomes[7] and syntheses of new anthracycline analogs.[8,9] New derivatives lacking the C_4 methoxy group have attracted much pharmacological and clinical interest, as these analogs share the properties of having a strong antitumor activity with reduced cardiotoxicity.[10,11] 4-Demethoxydaunorubicin (4DD, Idarubicin, I) (Fig. 1) is the most important member of the class of 4-demethoxy analogs and is currently being evaluated in phase I and II trials in patients with acute leukemia and a variety of solid tumors.[12–15] Apart from reduced cardiotoxicity, 4DD is distinguished from

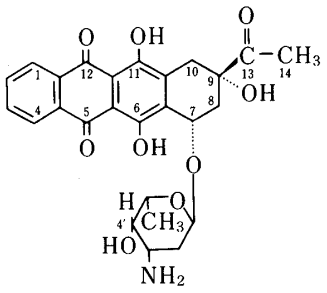

Fig. 1. Structure of 4DD

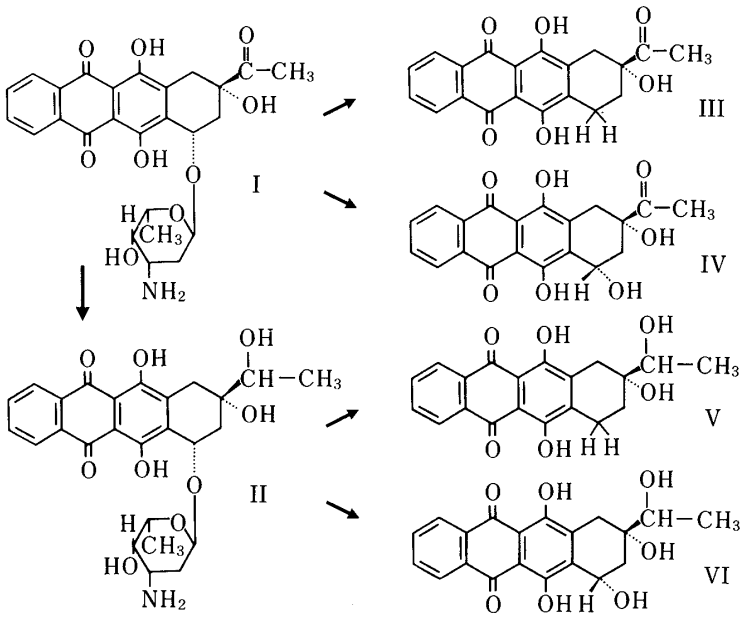

Fig. 2. Proposed Pathways for 4DD Metabolism

other anthracyclines in that it is orally active.[16,17] Anthracyclines are metabolized to a great extent. The C_{13} hydroxy metabolites are potent, while the aglycone metabolites lack antitumor activity but are suspects in eliciting cardiotoxicity.[18] This implies that, in order to obtain a full picture of the pharmacokinetic profile and metabolic fate of the drug, an analytical method is required which is capable of analyzing 4DD, 4-demethoxydaunorubicinol (4DD-ol, II) and the potential aglycone metabolites (Fig. 2) in low amounts, preferentially in small volumes of samples. Reported HPLC methods for 4DD analysis do not meet all the requirements.[15,19-22] We designed an HPLC assay capable of analyzing 4DD, 4DD-ol, four possible aglycone metabolites and the internal standard Dx in a 15-min isocratic run. The assay was tested in a pharmacokinetic study with two rabbits.[23]

Materials and Methods

Chemicals——4DD, 4DD-ol and Dx were generous gifts from Dr. S. Penco, Farmitalia Carlo Erba (Milan, Italy). The 7-hydroxy aglycones, 4-demethoxydaunorubicinone (IV) and 4-demethoxy daunorubicinolone (VI) were prepared by acidic hydrolysis of 4DD and 4DD-ol, respectively, and subsequent chloroform extraction as described previously.[24] 4-Demethoxy-7-deoxydaunorubicinone (III) and 4-demethoxy-7-deoxydaunorubicinolone (V) were prepared by controlled potential electrolysis at -750 mV.

Electrochemistry——Direct current (DC) polarography and controlled potential electrolysis (CPE) were performed using a Bruker E310 modular electrochemical system, equipped with a droptimer and a Houston model 2000 X-Y recorder (DC) or a Kipp BD-41 y-t recorder (CPE). A water-jacketed 10 ml polarographic cell (Metrohm EA 880-T-5) with a mercury pool electrode (approx. 7 cm^2), a Metrohm EA 436 Ag/AgCl/3 M KCl reference electrode and a platinum wire auxiliary electrode were employed. CPE was carried out in 10 ml of a 1×10^{-4} M solution of 4DD or 4DD-ol in 0.1 M phosphate buffer, pH 7. The desired potential of electrolysis was chosen from a DC polarographic curve which was recorded first. The course of the electrolysis was monitored at appropriate time intervals by DC polarography as well as high-performance liquid chromatography (HPLC). After the electrolysis has been completed the 7-deoxy aglycone products were extracted from the reaction solutions with chloroform.

Determination of pK_a Values of 4DD——The ionization constants of 4DD were determined spectrophotometrically at 570 nm and calculated according to the procedures described by Sturgeon and Schulman for Dx.[25] The concentration of 4DD was 2.2 mg/ml. Absorbance measurements were performed using a Shimadzu UV-140 double beam spectrophotometer (Shimadzu Corp. Kyoto, Japan).

HPLC——The chromatographic system consisted of a solvent delivery system, type 6000 A and an automatic sample injection device WISP, model 710 (both from Waters Assoc., Milford, MA, U.S.A.). Separation was performed on a Lichrosorb RP-8 (5 μm) column (125 mm × 4 mm i.d., Merck, Darmstadt, F.R.G.). To protect the

analytical column, a pre-column (40 mm × 4 mm i.d.), dry-packed with Lichrosorb RP-8 (20—100 μm) material (Merck, Darmstadt, F.R.G.) was used. The pre-column material was replaced weekly. The column effluent was subjected to fluorescence detection using a Perkin-Elmer LS1 fluorescence detector with excitation at 460 nm and monitoring the emission at 540 nm. Retention times and peak areas were measured with an SP-4000 (Spectra Physics, Santa Clara, CA, U.S.A.) data system.

Sample Pretreatment——For the isolation of 4DD and 4DD-ol as well as the aglycone metabolites from plasma or urine, a 0.1 or 1.0 ml sample, respectively, was mixed with 1.0 or 5.0 ml of chloroform–1-propanol (4 : 1, v/v) and 100 μl of 0.4 M borate buffer, pH 9.25, or 100 μl of 0.4 M borate buffer, pH 10.0, respectively. Subsequently, the internal standard Dx (120 ng) was added. After vortex mixing for 30 s, and centrifugation for 5 min (2500 × *g*) the supernatant and protein interface were discarded by suction under reduced pressure. Next, the resulting clear organic phase was evaporated to dryness under a gentle stream of nitrogen. The residue was dissolved in 100 μl of methanol and aliquots of 10—40 μl were injected into the chromatograph.

Calibration——Plasma or urine (0.1 or 1.0 ml) in polypropylene tubes was spiked with appropriate amounts of 4DD and 4DD-ol. After mixing, the samples were treated as described under "Sample Pretreatment" for the appropriate biological matrix. Quantitation of 4DD and 4DD-ol was performed by linear regression analysis of the ratio of the 4DD or 4DD-ol peak areas to the peak area of the internal standard Dx. Due to the limited amounts of the aglycones available for this study it was not possible to set up reference standard curves of the aglycones. Consequently, the quantitative evaluation of the HPLC assay of these compounds has been omitted. Quantitation of the aglycone metabolites was, therefore, performed by using the 4DD calibration curves where the ratio 4DD/Dx were plotted *versus* the molar 4DD concentration. This policy is based on the assumption that a constant ratio exists between the molar fluorescence yield of the aglycones and the parent compounds.

Recovery——The recoveries of 4DD and 4DD-ol were determined as follows. Plasma and urine samples were spiked with 4DD and 4DD-ol and treated according to the described procedures, except for the addition of the internal standard. Next, the external standard Dx (100 ng) was added to 800 μl (for the 0.1 ml samples) or 4.00 ml (for the 1.0 ml samples) of the extraction fluid. After evaporation and reconstitution with 100 μl of methanol an aliquot was injected into the chromatograph. The recoveries were calculated from the peak area ratios of 4DD and 4DD-ol with Dx and the peak ratios obtained after injection of a solution containing 80 ng of 4DD, 80 ng of 4DD-ol and 100 ng of Dx per 100 μl of methanol. The recovery of Dx has been determined in the same way, using 4DD as an external standard.

Precision——The precision of the method was determined by assaying plasma samples on three different days.

Detection Limit——For the establishment of the detection limit of the HPLC assay the fluorescence detector was set at a response equal to three times the average noise level.

Pharmacokinetic Experiments——Two rabbits (rabbit A, 2.6 kg; rabbit B, 2.9 kg) were given 5.5 mg of 4DD dissolved in 5.0 ml of saline, as a bolus injection (infusion time 40 s) in an ear vein. Periodically, until 96 h after injection, blood samples were withdrawn from the other ear and immediately centrifuged to obtain plasma samples. The blood samples were collected in polypropylene tubes containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. Urine was collected during 24-h periods. Plasma and urine samples were stored at −20 °C prior to analysis, two weeks after sampling.

Pharmacokinetic Analysis——Plasma concentration time curves were analyzed with the computer program PHARMLINE.[26] This least-squares, iterative program determines slopes and intercepts of the logarithmically plotted curves of multi-exponential functions. It provides correlation coefficients of the estimated curve. In the case of linear kinetics, plasma drug concentration at any time t after an intravenous bolus dose is given by:

$$c = \sum_{i=1}^{n} c_i \cdot e^{-\lambda_i x t}$$

where λ_i is the exponent of the i-th exponential term, c is the plasma concentration and c_i is the initial concentration of the i-th component of the curve. Total plasma clearance, calculated from the plasma curve is given by:

$$Cl = \text{dose}/AUC$$

The area under the curve (AUC) was calculated by means of the trapezoidal rule. Rate constants and volumes of distribution were calculated according to the appropriate equations by the computer program.

Results

Chromatography

HPLC analysis of anthracyclines, their metabolites and degradation products is usually performed using reversed-phase systems.[27] The chromatographic behavior of the anthraquinone glycosides is strongly influenced by variation of the pH and ionic strength of the mobile phase, at a constant water/organic modifier (mostly acetonitrile or methanol) ratio.

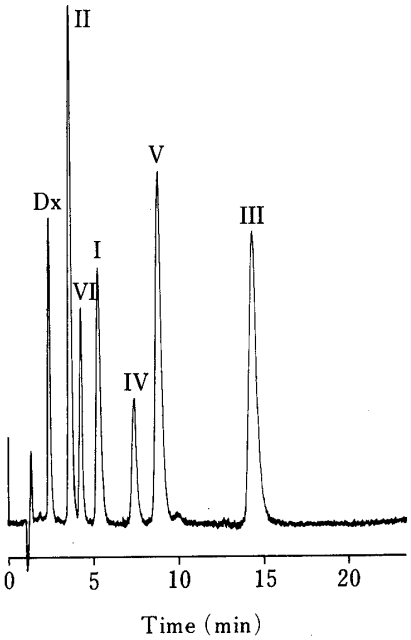

Fig. 3. HPLC Chromatogram of a Mixture of Reference Compounds

Dx = doxorubicin; I = 4DD; II = 4DD-ol; III = 4-demethoxy-7-deoxydaunorubicinone; IV = 4-demethoxydaunorubicinone; V = 4-demethoxy-7-deoxydaunorubicinolone; VI = 4-demethoxydaunorubicinolone.

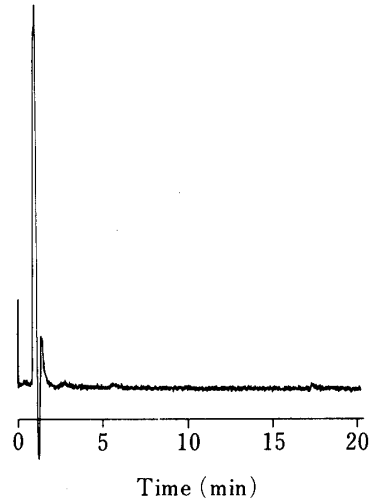

Fig. 4. HPLC Chromatogram of Untreated Rabbit Plasma

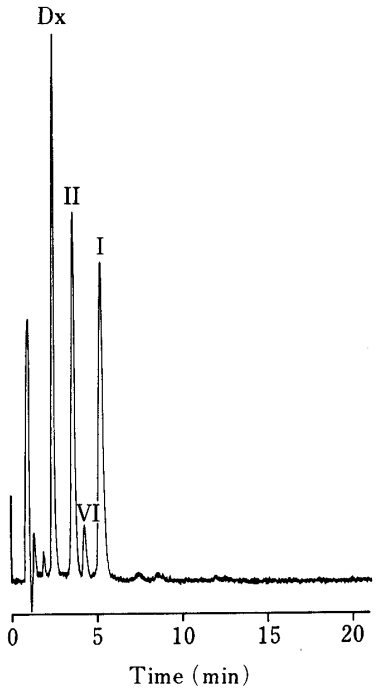

Fig. 5. HPLC Chromatogram of Plasma of Treated Rabbit ($t = 20$ min)

Fig. 6. HPLC Chromatogram of Untreated Rabbit Urine

This is in contrast to the chromatography of the aglycone compounds that depends only on the water/organic modifier ratio in the eluting solvent.[27] By variation of pH, ionic strength and organic modifier concentration of the mobile phase the optimal separation between 4DD

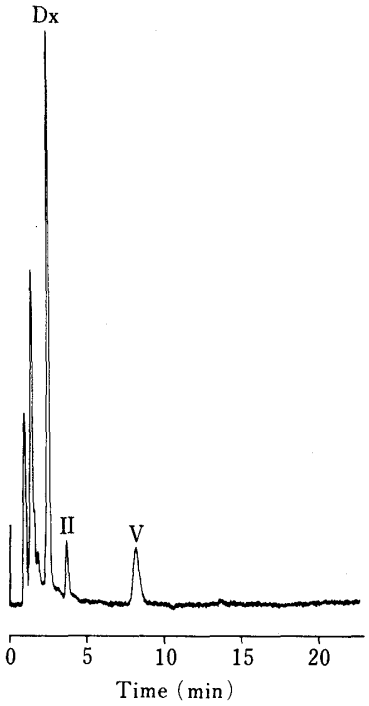

Fig. 7. HPLC Chromatogram of Urine of Treated Rabbit (24-h Urine Collected on Day 1)

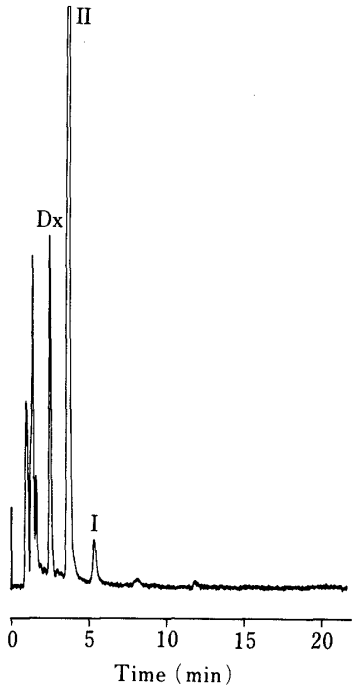

Fig. 8. HPLC Chromatogram of Urine of Treated Rabbit (24-h Urine Collected on Day 3)

and its potential metabolites could be achieved. An HPLC chromatogram of a mixture of reference compounds, including Dx, is depicted in Fig. 3. In this analysis acetonitrile–phosphate buffer, pH 2.4 (70 : 30, w/w) was used as the eluent. Several column materials have been tested and the Lichrosorb RP-8 (particle size, 5 μm) stationary phase was found to be suitable.

Representative HPLC chromatograms of plasma and urine blank samples and samples after treatment of the rabbits are shown in Figs. 4—8. The peaks in the chromatograms of the blank samples did not interfere with the compounds of interest.

Sample Pre-treatment Procedures

Blood samples were collected in tubes containing EDTA as it had been reported that Dx forms ionic complexes with heparin, interfering with the HPLC separation.[28,29] This can be expected to occur for 4DD as well. At physiological pH anthraquinone glycosides exist almost exclusively as monocations, due to protonation of the 3′-amino function. The positively charged ions may interact electrostatically with the negatively charged mucopolysaccharide heparin. We found no indication that such interactions exist between EDTA and 4DD.

After sampling it is essential that the plasma fraction is separated immediately from the blood cells by centrifugation. Anthracyclines are rapidly concentrated in blood cells and then become a substrate for cytoplasmatic aldo-keto reductase enzymes converting the drugs into their respective C_{13} hydroxyl metabolites.[30]

Isolation of anthraquinone glycosides and metabolites from biological matrices can be accomplished in several ways. Liquid–liquid extraction is usually preferred, although liquid–solid extraction is a good alternative.[27] The extraction properties of Dx, daunorubicin and their C_{13} hydroxyl metabolites have been investigated in detail by Eksborg.[31] For optimal extraction efficacies the pH of the aqueous phase is very important. 4DD can exist in five different forms connected with the protonation degree of the molecule. The different species are: monocation (protonated 3′-amino function), neutral species, zwitterion (protonated 3′-

amino function and deprotonated phenolic group), mono-anion (deprotonated phenolic function) and di-anion (both phenolic groups deprotonated). Only the uncharged species are extractable. The fractions of these species, as functions of pH, can be calculated from Eq. 1:

$$f^0 = [H^+]^2 K_1 / ([H^+]^3 + [H^+]^2 K_1 + [H^+] K_1 K_2 + K_1 K_2 K_3) \quad (1)$$

The pK values, determined spectrophotometrically, appear to be: $pK_1 = 8.2$, $pK_2 = 10.1$ and $pK_3 = 13.2$. From Eq. 1 it is clear that the extraction of 4DD is optimal around pH 9. The amount of buffer added to the biological samples was adjusted so that the final pH of the solution reached 9. In order to obtain high recoveries, the presence of 1-pentanol in the extraction solvent appears to be essential. After extraction of the plasma samples with chloroform–1-heptanol by the method of Moro *et al.*, the extract was reextracted with 0.3 M phosphoric acid.[22] This acidic phase then was analyzed by HPLC. With this procedure the aglycones are excluded from the chromatographic analysis as these compounds remain in the organic phase. In the procedure described here the organic extract is directly subjected to HPLC analysis, in order to include any possible aglycone metabolite in the assay. After evaporation of the organic solvent, the residue was reconstituted with methanol and this solution was subsequently injected into the chromatograph. The residue could not be completely re-dissolved if the mobile phase was used as reconstitution fluid. Methanol samples up to 40 μl could be injected without interference in the chromatography. Injection of larger amounts of methanol caused disturbance of the chromatographic system, resulting in peak broadening.

Evaluation of the Analytical Methodology

The recoveries of 4DD and 4DD-ol in the concentration range of 10—1000 ng/ml (using 0.1 or 1.0 ml plasma or urine samples) were always better than 90%, with a relative standard deviation (RSD) of 5—12%. The day-to-day RSD of the assay at a plasma concentration of 100 ng/ml was 6%. Standard curves from spiked samples were linear over the working concentration range of 1—1000 ng/ml ($r > 0.999$). The detection limit for 4DD and 4DD-ol was 1 ng/ml (using 1.0 ml samples).

4DD plasma samples could be stored at -20°C for a period of two weeks without any loss.

Pharmacokinetics

The principal metabolite in plasma and urine of the rabbit appeared to be 4DD-ol.

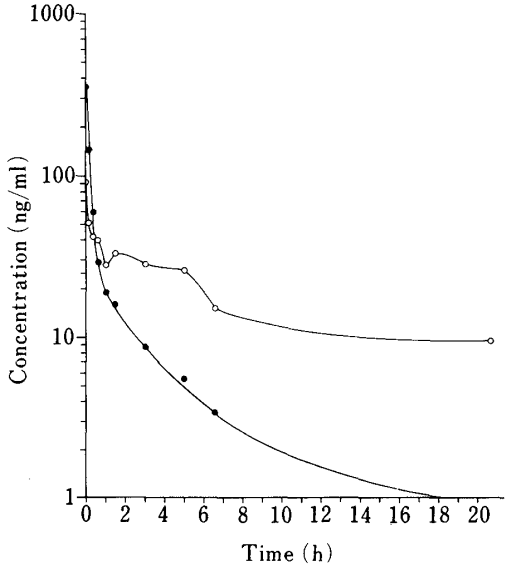

Fig. 9. log Plasma Concentration–Time Curves for Rabbit A

●, 4DD; ○, 4DD-ol.

TABLE I. The 24 h Volumes of Urine Produced by the Rabbits and Concentration of Compounds Therein

Day	Volume (ml)	[4DD] (ng/ml)	[4DD-ol] (ng/ml)	[V][a] (ng/ml)
Rabbit A				
1	160	63	900	20
2	145	17	230	23
3	140	—	62	81
4	160	—	—	88
Rabbit B				
1	260	60	426	19
2	105	11	155	22
3	140	—	—	132
4	300	—	—	63

a) Based on the assumption of a constant molar ratio of fluorescence yield between *V* and 4-DD.

TABLE II. Pharmacokinetic Parameters of the log-Mean Plasma Concentration *versus* Time Curves from Rabbits A and B, after Injection of 5.5 mg of 4DD

Parameter	4DD		4DD-ol	
	2 comp	3 comp	2 comp	3 comp
c_1 (ng/ml)	216	427	54.4	70.2
c_2 (ng/ml)	9.63	32.6	23.9	17.6
c_3 (ng/ml)	—	5.54	—	20.4
λ_1 (h^{-1})	3.73	9.43	2.84	9.88
λ_2 (h^{-1})	0.072	0.538	0.048	0.42
λ_3 (h^{-1})	—	0.057	—	0.046
V_1 (l)	24	12	70	51
V_2 (l)	257	46	143	82
V_3 (l)	—	184	—	88
Cl (l/h)	29	27	11	11
AUC (mg·h/l)	192	202	513	492
k_{12} (h^{-1})	2.397	4.796	1.834	5.855
k_{21} (h^{-1})	0.228	1.227	0.899	3.616
k_{13} (h^{-1})	—	1.604	—	0.416
k_{31} (h^{-1})	—	0.103	—	0.24
k_{10} (h^{-1})	1.178	2.295	0.153	0.22
t_2 a (h)	0.19	0.07	0.24	0.07
t_2 b (h)	9.62	1.23	14.3	1.65
t_2 c (h)	—	12.1	—	15.1
Corr. coef.	0.741	0.990	0.920	0.969

c_1—c_3, plasma concentrations at $t=0$ of the 1st to 3rd compartments; λ_1—λ_3, exponents of the respective exponential terms; V_1—V_3, volumes of distribution; *Cl*, clearance; *AUC*, the area under the curve; k_{12}—k_{10}, rate constants.

The concentration of this metabolite exceeds that of the parent drug (Fig. 9). In plasma the aglycone VI (4-demethoxydaunorubicinolone) is the only other metabolite appearing. In urine samples a peak with the same retention time as V (4-demethoxy-7-deoxy-daunorubicinolone) was observed. The concentrations of the compounds found in the 24-h urine samples are listed in Table I. The log concentration–time curves for 4DD and 4DD-ol in plasma were evaluated using two- and three-compartment models. The pharmacokinetic data are shown in Table II. It can be seen that a three-compartment model fits better for the parent compound and 4DD-ol than a two-compartment model. The correlation

coefficients for a two-compartment model are 0.74 and 0.92 for 4DD and 4DD-ol, respectively, and for the three compartment model these values are 0.99 and 0.97. The plasma clearance of 4DD is 27 l/h, while the clearance of the C_{13} hydroxy metabolite is only 11 l/h. The clearance of 4DD-ol was calculated based on the dose of 4DD (5.5 mg). The total volumes of distribution are 242 and 221 l for 4DD and 4DD-ol, respectively, suggesting that the distribution of the metabolite is similar to that of its parent compound, although the central compartment of 4DD-ol is 4 times larger than that of 4DD.

4DD-ol is very rapidly formed and was already present in the first plasma sample. The plasma levels of 4DD-ol increase from 30 min to 3 h after injection, indicating that an enterohepatic circulation of this compound occurs (Fig. 9).

Discussion

In this paper an HPLC determination method for the analysis of 4DD and metabolites in plasma and urine samples is presented. The HPLC system and sample pre-treatment procedures are based upon methods for Dx and daunorubicin published before.[31] The pharmacokinetic data and metabolic profile of 4DD are in reasonable agreement with the results obtained earlier for daunorubicin. Oosterbaan *et al.*, who studied structure-related body distribution of anthracyclines in dogs, found similar plasma profiles of 4DD and 4DD-ol.[32] A three-compartment model also fitted their data best. The appearance of 4DD-ol in the first plasma sample, the plateau at 3 h after injection and the parallel declining plasma concentrations of both compounds suggest that metabolism is the rate-limiting step in the clearance process of 4DD-ol. The clearance of 4DD-ol, as noted in Table II, probably represents the metabolic clearance by the liver. The intrinsic clearance of the C_{13} hydroxyl metabolite 4DD-ol will be much higher. A pharmacokinetic study with this compound will be required to confirm these points. We conclude that this method of analysis gives reliable data which can be used for adequate pharmacokinetic analysis.

References and Notes

1) H. L. Davis and T. E. Davis, *Cancer Treat. Rep.* **63**, 809 (1979).
2) J. R. Brown and S. H. Imam, *Progr. Med. Chem.*, **21**, 170 (1984).
3) A. Di Marco, *Drugs Exptl. Clin. Res.*, **9**, 751 (1983).
4) F. Arcamone, "Doxorubicin Anticancer Antibiotics," Academic Press, New York, 1981.
5) E. G. Mimnaugh, Z. H. Siddik, R. Drew, B. I. Sikic and T. E. Gram, *Toxicol. Appl. Pharmacol.*, **49**, 119 (1979).
6) B. L. Lum, J. M. Svec and F. M. Torti, *Drug Intell. Clin. Pharm.*, **19**, 259 (1985).
7) E. H. Herman, A. Rahman, V. J. Ferrans, J. A. Vick and P. S. Schein, *Cancer Res.*, **43**, 5427 (1983).
8) F. Arcamone, *Cancer Res.*, **45**, 5995 (1985).
9) A. M. Casazza, *Cancer Treat. Rep.*, **63**, 835 (1979).
10) S. Penco, A. M. Casazza, G. Franchi, B. Barbieri, O. Bellini, A. Podesta, G. Savi, G. Pratesi, G. Geroni, A. Di Marco and F. Arcamone, *Cancer Treat. Rep.*, **67**, 665 (1983).
11) R. Supino, A. Necco, T. Dasdia, A. M. Casazza and A. Di Marco, *Cancer Res.*, **37**, 4523 (1977).
12) S. Kaplan, C. Sessa, Y. Willems, M. A. Pacciarini, V. Tamassia and F. Cavalli, *Invest. New Drugs*, **2**, 281 (1984).
13) V. Bonafante, L. Ferrari, F. Villani and G. Bonadonna, *Invest. New Drugs*, **1**, 161 (1983).
14) S. Kaplan, A. Martini, M. Varini, P. Togni and F. Cavalli, *Eur. J. Cancer Clin. Oncol.*, **18**, 1303 (1982).
15) A. N. Daghestani, Z. A. Arlin, B. Leyland-Jones, T. S. Gee, S. J. Kempin, R. Mertelsmann, D. Budman, P. Schulman, R. Baratz, L. Williams, B. D. Clarkson and C. W. Young. *Cancer Res.*, **45**, 1408 (1985).
16) A. Di Marco, A. M. Casazza and G. Pratesi, *Cancer Treat. Rep.*, **61**, 893 (1977).
17) A. Martoni, M. A. Pacciarini and F. Pannuti, *Eur. J. Cancer Clin. Oncol.*, **21**, 803 (1985).
18) R. Mhatre, E. Herman, A. Huidobro and V. Waravdekar, *J. Pharmacol. Exp. Ther.*, **178**, 216 (1971).
19) M. J. M. Oosterbaan, R. J. M. Dirks, T. B. Vree and E. van der Kleijn, *J. Chromatogr.*, **306**, 323 (1984).
20) G. Pizzorno, F. Trave, A. Mazzoni, O. Russello and A. Nicolin, *J. Liq. Chromatogr.*, **8**, 2557 (1985).
21) K. Lu, N. Savaraj, J. Kavanagh, L. G. Feun, M. Burgess, G. P. Bodey and T. L. Loo, *Cancer Chemother. Pharmacol.*, **17**, 143 (1986).

22) E. Moro, V. Bellotti, M. G. Jannuzzo, S. Stegnjaich and G. Valzelli, *J. Chromatogr.*, **274**, 281 (1983).
23) J. H. Beijnen, S. Rodenhuis, A. C. A. Paalman and W. J. M. Underberg, *Proc. Am. Assoc. Cancer Res.*, **28**, 434 (1987).
24) J. H. Beijnen, G. Wiese and W. J. M. Underberg, *Pharm. Weekbl. Sci. Ed.*, **7**, 109 (1985).
25) R. J. Sturgeon and S. G. Schulman, *J. Pharm. Sci.*, **66**, 958 (1977).
26) F. Rombout, N. C. Punt, J. Boiten and D. K. F. Meijer, Abstracts of Papers, 47th International Congress of Pharmaceutical Sciences of F. I. P., Amsterdam, August September 1987, p. 73.
27) J. Bouma, J. H. Beijnen, A. Bult and W. J. M. Underberg, *Pharm. Weekbl. Sci. Ed.*, **8**, 109 (1986).
28) E. Confrancesco, A. Vigo and E. Pogliani, *Thrombosis Res.*, **18**, 743 (1980).
29) J. Cummings, J. F. B. Stuart and K. C. Calman, *J. Chromatogr.*, **311**, 125 (1984).
30) D. H. Huffman and N. R. Bachur, *Cancer Res.*, **32**, 600 (1972).
31) S. Eksborg, *J. Pharm. Sci.*, **67**, 782 (1978).
32) M. J. M. Oosterbaan, R. J. M. Dirks, T. B. Vree and E. van der Kleijn, *Pharm. Res.*, **1**, 33 (1984).