

Extraction of Daunorubicin and Doxorubicin and Their Hydroxyl Metabolites: Self-Association in Aqueous Solution

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Abstract □ The extraction of daunorubicin and doxorubicin and their hydroxyl metabolites daunorubicinol and doxorubicinol was studied using chloroform-1-pentanol (9:1) as the organic phase. Because of differences in acid dissociation constants, the pH for optimum extraction varied from 8.0 to 8.6 for the different compounds. Self-association in the aqueous phase significantly influenced the distribution ratio. Constants for the formation of dimers and tetramers in aqueous solutions were about $10^{4.5}$ and 10^{12} , respectively.

Keyphrases □ Daunorubicin—organic-aqueous distribution, self-association in aqueous phase □ Doxorubicin—organic-aqueous distribution, self-association in aqueous phase □ Distribution, organic-aqueous—daunorubicin and doxorubicin and hydroxyl metabolites □ Antineoplastic agents—daunorubicin, doxorubicin, and hydroxyl metabolites, organic-aqueous distribution, self-association in aqueous phase

Daunorubicin (I) and doxorubicin (II) are cytotoxic antibiotics used for the treatment of leukemia and solid tumors (1–3). Their cytotoxic effect has been attributed to complex formation with DNA resulting in an inhibition of RNA and DNA syntheses (4–8). The main metabolites of daunorubicin and doxorubicin, daunorubicinol (III) and doxorubicinol (IV), respectively, also have cytotoxic activity (8–10).

Self-association in aqueous solutions has been reported for planar aromatic ring systems (11–14). Dimerization of I in aqueous solutions has been studied by various physicochemical methods (photometry, circular dichroism, and NMR spectroscopy) (15). The self-association of I and II was investigated by studying its effect on the absorption spectrum and the distribution ratio to an organic phase.

The optimum conditions for extraction of I and II and their metabolites can be calculated from the constants presented.

EXPERIMENTAL

All experiments were carried out at $25.0 \pm 0.1^\circ$.

Chemicals—Doxorubicin¹, doxorubicinol¹, daunorubicin², and daunorubicinol² were used as obtained. Chloroform was extracted with water to remove ethanol.

Aqueous and organic phases were equilibrated carefully before use. All other chemicals and solvents were analytical grade.

Photometric Measurements—The photometric determinations³ of acid dissociation constants were based on the following measurements.

Stock solutions of I and II in distilled water were diluted with pH 9.1–10.5 carbonate buffers, 10^{-1} M phosphoric acid, or 10^{-2} M sodium hydroxide. The absorbance at 480 nm was measured within 60 sec after dilution to avoid decomposition of the components. The slit width of the photometer was kept constant within each series. The absorbance of at least two dilutions was measured at each pH value⁴.

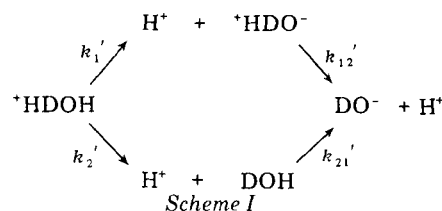
Partition Experiments—The partition experiments were performed in centrifuge tubes using equal phase volumes (10–20 ml) and mechanical shaking for 30 min in a thermostated bath. After centrifugation, the phases were separated by a capillary siphon.

The concentrations of the components were determined fluorometrically⁵ (436 nm/555 nm) or photometrically (253 nm) in both phases: in the aqueous phase by direct measurement⁶ and in the organic phase after reextraction to 0.1 M H_3PO_4 . The spectrophotofluorometer was standardized against a solution of daunorubicin in 0.1 M H_3PO_4 before each measurement.

RESULTS AND DISCUSSION⁷

Photometric Determination of Acid Dissociation Constants—Compounds I and II have identical absorption spectra, which change drastically from acidic to basic media because of the protolysis of the two phenolic groups (Fig. 1). Studies of absorption spectra revealed that the acid dissociation constant of the second phenolic group was $<10^{-12}$. The exact value of the constant could not be established because of the low stability of the compounds in strongly alkaline media.

In the pH range where the dissociation of the second phenolic group is negligible (pH <11), the protolysis of the compounds can be illustrated by Scheme I.



Four forms of the compounds occur: the ammonium (${}^+HDOH$), the phenolate (DO^-), the uncharged (DOH), and the zwitterion (${}^+HDO^-$). The microscopic constants (16), k_1' , k_2' , k_{12}' , and k_{21}' , are defined by:

$$k_1' = \frac{a_h[{}^+HDO^-]}{[{}^+HDOH]} \quad (\text{Eq. 1})$$

$$k_2' = \frac{a_h[DOH]}{[{}^+HDOH]} \quad (\text{Eq. 2})$$

$$k_{12}' = \frac{a_h[DO^-]}{[{}^+HDO^-]} \quad (\text{Eq. 3})$$

$$k_{21}' = \frac{a_h[DO^-]}{[DOH]} \quad (\text{Eq. 4})$$

Photometric determination of the microscopic constants with graphic computation according to Edsall *et al.* (16), based on determined concentrations of species with protolyzed and unprotolyzed phenolic groups⁸, is illustrated in Fig. 2. Plots of α_{OH} as a function of pM_{OH}^* , where:

$$\alpha_{OH} = \frac{C_{DO^-}}{C_D} \quad (\text{Eq. 5})$$

⁵ Aminco-Bowman 4-8202 B spectrophotofluorometer.

⁶ Molar absorptivity at 253 nm was 2.1×10^4 (pH 1.0–7.5).

⁷ The following symbols, not defined in the text, are used: $\{ \}$ and $[]_{org}$ are the concentrations of molecules and ions in aqueous and organic phases, respectively; C_{DO^-} is the total concentration of the species in the aqueous phase containing a protolyzed phenolic group; C_{DOH} is the total concentration of the species in the aqueous phase containing unprotolyzed phenolic groups; $C_D = C_{DO^-} + C_{DOH}$ is the total concentration in the aqueous phase; and $C_{D,org}$ is the total concentration in the organic phase.

⁸ $C_{DOH}/C_{DO^-} = (A_B - A)/(A - A_A)$, where A , A_A , and A_B are the absorbances at 480 nm in buffer, 0.1 M H_3PO_4 , and 10^{-2} M NaOH, respectively (16).

¹ Supplied by Farmitalia, Milan, Italy.

² Supplied by Pharma Rhodia, Stockholm, Sweden.

³ The photometers used were a Zeiss PMQ III with 10.0- and 50.0-mm cells and a Pye Unicam model 1800 with 10.0-mm cells.

⁴ Orion Research model 701 digital pH meter equipped with an Ingold combined electrode type 401.

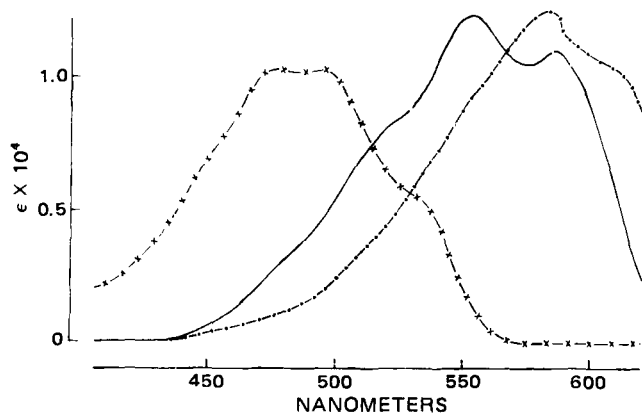


Figure 1—Absorption spectra of I. Key: ×, pH 2.0 and 7.5; —, pH 10.9; and ●, pH 14 (1 M NaOH). The total concentration was 10^{-5} M.

and:

$$M_{OH^*} = \frac{a_h C_{DO^-}}{C_{DOH}} \quad (\text{Eq. 6})$$

showed a variation of pM_{OH^*} not only with α_{OH} but also with C_D at constant values of α_{OH} . Hence, the protolysis of the phenolic groups is not only affected by the pH of the aqueous solutions but also by equilibrium processes dependent on the concentration of the drugs, e.g., formation of aggregates. Therefore, the results presented in Fig. 2 cannot be used directly for the determination of the microscopic constants.

At constant pH, the ratio C_{DOH}/C_{DO^-} increased with increasing total concentration, which will be the case when ${}^+HDOH$ and DOH are involved in association processes (e.g., dimerization and tetramerization) to a greater extent than ${}^+HDO^-$ and DO^- . Experiments were carried out in the pH range where the ${}^+HDOH$ form is unlikely. Under the assumptions that C_{DO^-} includes only monomeric forms:

$$C_{DO^-} = [{}^+HDO^-] + [DO^-] \quad (\text{Eq. 7})$$

and that C_{DOH} includes the monomer and dimer of the noncharged form:

$$C_{DOH} = [DOH] + 2[(DOH)_2] \quad (\text{Eq. 8})$$

the following equation is valid:

$$\frac{C_{DOH}}{C_{DO^-}} = \frac{[DOH]}{C_{DO^-}} + \frac{2[(DOH)_2]}{C_{DO^-}} \quad (\text{Eq. 9})$$

which, by substitution of the dimerization constant $K_{2(DOH)}$ defined by:

$$K_{2(DOH)} = \frac{[(DOH)_2]}{[DOH]^2} \quad (\text{Eq. 10})$$

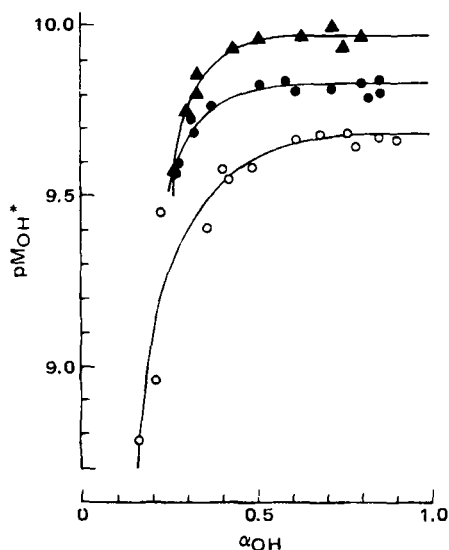


Figure 2—Photometric determination of microscopic constants for I. Key (total concentration): ▲, 2.125×10^{-4} M; ●, 1.063×10^{-4} M; and ○, 2.503×10^{-5} M.

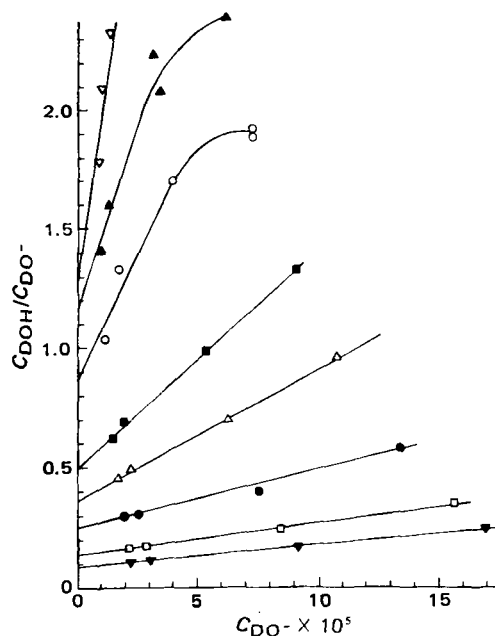


Figure 3—Photometric determination of acid dissociation constants and dimerization constant of I. The total concentration was $(0.250-2.125) \times 10^{-4}$ M in carbonate buffer, $\mu = 0.1$. Key (pH): ▼, 10.56; □, 10.42; ●, 10.18; ▲, 9.99; ■, 9.83; ○, 9.54; ▲, 9.38; and ▽, 9.14.

and:

$$M_{OH} = \frac{a_h([{}^+HDO^-] + [DO^-])}{[DOH]} = \frac{a_h C_{DO^-}}{[DOH]} \quad (\text{Eq. 11})$$

gives:

$$\frac{C_{DOH}}{C_{DO^-}} = \frac{a_h}{M_{OH}} + \frac{2K_{2(DOH)}a_h^2 C_{DO^-}}{M_{OH}^2} \quad (\text{Eq. 12})$$

In the pH 9.8–10.5 range, plots of C_{DOH}/C_{DO^-} versus C_{DO^-} at constant pH gave straight lines (Fig. 3), supporting the validity of Eq. 12. Deviations from linearity were obtained at lower pH (9.1–9.5) values, with the increase of C_{DOH}/C_{DO^-} declining at higher C_{DO^-} . This decline may be the result of an association process involving ${}^+HDO^-$ (e.g., dimerization of ${}^+HDO^-$), while formation of higher aggregates than dimers of DOH , e.g., tetramers, results in a deviation from linearity with a steepening of the curve with increasing C_{DO^-} .

Calculations from intercepts of plots in the pH 9.4–10.5 range according to Eq. 12 showed that pM_{OH} did not vary with α_{OH} (range of 0.4–0.95). Therefore, it can be concluded that formation of the zwitterion in the monomeric form is negligible; hence, $k_2' \gg k_1'$ (16).

For both I and II, $pM_{OH} = pk_{21}' = 9.54 \pm 0.03$, i.e., the acid dissociation constant of the first phenolic group is not affected by the addition of an alcoholic group in the side chain. The obtained value of pk_{21}' also probably is valid for III and IV. For I and II, $\log K_{2(DOH)} = 4.2$ and 4.3, respectively, was obtained in the pH 9.8–10.5 range from slopes of plots according to Eq. 12.

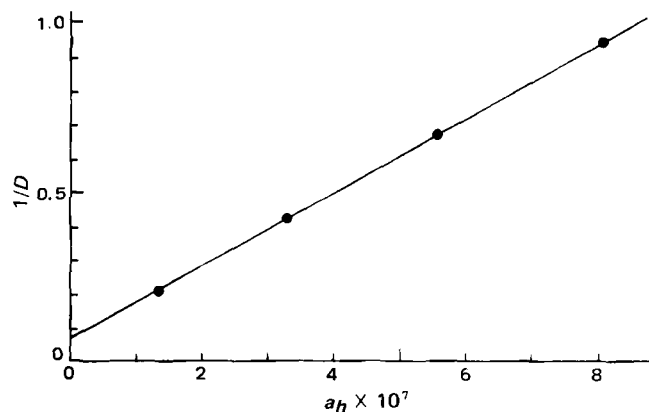


Figure 4—Evaluation of k_d and k_2' for II. (Conditions were as given in Table I.)

Table I—Partition Coefficients

Compound	pH	$C_{aq} \times 10^7$ ^a	$C_{org} \times 10^7$ ^b	$-\log k_d k_2' \pm SEM$	$\log k_d \pm SEM$	$pk_2' \pm SEM$
I	4.34–5.78	0.35–7.5	0.57–22.9	4.90 ± 0.01	1.5 ± 0.1	6.4 ± 0.1
III	5.85–6.69	0.37–1.36	1.66–17.4	5.65 ± 0.01	0.99 ± 0.07	6.64 ± 0.08
II	5.55–6.86	0.34–16.5	0.53–13.8	6.03 ± 0.01	1.17 ± 0.05	7.20 ± 0.05
IV	6.47–7.22	9.44–12.5	1.11–4.22	7.48 ± 0.01	0.21 ± 0.09	7.69 ± 0.09

^a Aqueous phase: pH > 5.0, phosphate buffer, $\mu = 0.1$; and pH < 5.0, citrate buffer, $\mu = 0.1$. ^b Organic phase was chloroform-1-pentanol (9:1).

Table II—Dimerization and Tetramerization Constants

Compound	pH	$C_{aq} \times 10^4$ ^a	$C_{org} \times 10^5$ ^b	$\log K_{(2)} \pm SE$	$\log K_{(4)} \pm SE$	$\log K_{2(DOH)} \pm SE$ ^c
I	4.34–4.88	0.43–5.49	0.65–5.71	4.48 ± 0.06	12.0 ± 0.1	4.2 ± 0.1
II	5.78–6.20	0.18–3.26	0.80–9.55	4.49 ± 0.02	12.3 ± 0.2	4.3 ± 0.1

^a Aqueous phase: pH > 6.0, phosphate buffer, $\mu = 0.1$; and pH < 6.0, citrate buffer, $\mu = 0.1$. ^b Organic phase was chloroform-1-pentanol (9:1). ^c Determined photometrically (aqueous solution system) at pH 9.8–10.5 (carbonate buffer, $\mu = 0.1$), $C_D = (0.25-2.1) 10^{-4} M$.

Distribution Coefficients—Fluorescence and absorption spectra of I extracted into chloroform-1-pentanol (9:1) from a pH 7 buffer and an aqueous solution of I at pH 2 showed good agreement in location and intensity of maxima. Therefore, it can be assumed that this type of drug is extracted in the uncharged form, *i.e.*, DOH (17). Under the assumption that only monomeric forms are present, the distribution ratio, *D*, can be written as:

$$D = \frac{C_{D,org}}{C_D} = \frac{k_d}{a_h/k_2' + k_1'/k_2' + k_2'/a_h + 1} \quad (\text{Eq. 13})$$

where the distribution coefficient, k_d , is defined by:

$$k_d = \frac{[\text{DOH}]_{org}}{[\text{DOH}]} \quad (\text{Eq. 14})$$

Optimal extraction is obtained when $\text{pH} = (\text{pk}_2' + \text{pk}_{21}')/2$.

When $\text{pH} \leq \text{pk}_{21}' - 1$, Eq. 13 can be transformed to:

$$\frac{1}{D} = \frac{1}{k_d} \left(1 + \frac{k_1'}{k_2'} \right) + \frac{a_h}{k_d k_2'} \quad (\text{Eq. 15})$$

monomeric forms occur. The pH for optimal extraction varies for the different compounds, *e.g.*, $\text{pH}_{opt} = 8.0$ for I and 8.6 for IV, and is independent of the organic solvent used for the extraction. This fact must be taken into account in the extraction of the unchanged drugs and their metabolites from biological samples (19–24).

In the pH 4.3–6.2 range, the distribution ratio of I and II decreases with increasing concentration at constant pH ($C_{aq} > 10^{-5} M$). Generally, this will be the case when association formation (*e.g.*, dimerization and tetramerization) occurs to a higher extent in the aqueous than in the organic phase.

In this pH range, however, monomers of I and II can be assumed to be present mainly in the forms of ⁺HDOH and DOH. Dimers and tetramers probably will have the compositions (⁺HDOH)_{*n*}·(DOH)_{*2-n*} and (⁺HDOH)_{*m*}·(DOH)_{*4-m*}, respectively, where *n* and *m* are the numbers of positively charged species in the dimers and tetramers formed. The negatively charged species are present in too low concentration to form dimers and tetramers. Under the assumption that only the monomer, DOH_{org}, is present in the organic phase, the following equation is valid:

$$D = \frac{[\text{DOH}]_{org}}{[\text{+HDOH}] + [\text{DOH}] + 2[(\text{+HDOH})_n(\text{DOH})_{2-n}] + 4[(\text{+HDOH})_m(\text{DOH})_{4-m}]} \quad (\text{Eq. 16})$$

Equation 16 can be given the form:

$$\frac{1}{D} = a_0 + a_1 C_{D,org} + a_2 C_{D,org}^3 \quad (\text{Eq. 17})$$

where:

$$a_0 = \frac{1}{k_d} + \frac{a_h}{k_d k_2'} \quad (\text{Eq. 18})$$

$$a_1 = \frac{2K_{(2)} a_h^n}{k_d^2 k_2'^n} \quad (\text{Eq. 19})$$

$$a_2 = \frac{4K_{(4)} a_h^m}{k_d^4 k_2'^m} \quad (\text{Eq. 20})$$

The dimerization and tetramerization constants, $K_{(2)}$ and $K_{(4)}$, respectively, are defined by:

$$K_{(2)} = \frac{[(\text{+HDOH})_n(\text{DOH})_{2-n}]}{[\text{+HDOH}]^n [\text{DOH}]^{2-n}} \quad (\text{Eq. 21})$$

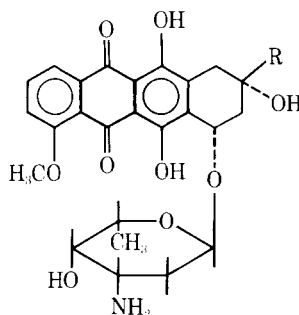
$$K_{(4)} = \frac{[(\text{+HDOH})_m(\text{DOH})_{4-m}]}{[\text{+HDOH}]^m [\text{DOH}]^{4-m}} \quad (\text{Eq. 22})$$

and a_0 , a_1 , and a_2 were estimated from $1/D$ and $C_{D,org}$ at constant pH by multiple linear regression.

The relative amounts of monomer, dimer, and tetramer were approximately 13–44, 55–67, and 1–20%, respectively, for I and 22–59, 40–56, and 1–22%, respectively, for II, calculated from the estimates of a_0 , a_1 , and a_2 and the total concentration in the aqueous phase. Under these experimental conditions, high accuracy can be expected for the estimates (25). Due to the few experimental points at each pH ($N = 9-10$), the precision of the estimates was rather low.

The values of $k_d k_2'$ calculated from plots of a_0 versus a_h were in agreement with values presented in Table I, but the precision was considerably lower (± 0.2 log unit, *SE*).

Plots of a_1 versus a_h^2 and a_2 versus a_h^4 gave straight lines for both I and II, indicating that the dimers and tetramers formed were composed



- I: R = C(=O)CH₃
- II: R = C(=O)CH₂OH
- III: R = CH(OH)CH₃
- IV: R = CH(OH)CH₂OH

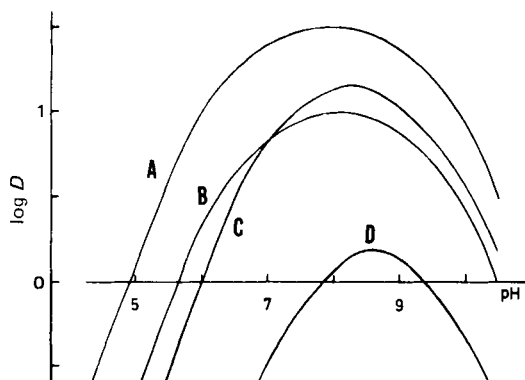


Figure 5—Variation of the distribution ratio with pH of the aqueous phase. Calculations were based on constants given in Table I and $pK_{21}' = 9.54$. Key: A, I; B, III; C, II; and D, IV.

of positively charged species (Fig. 6). The values of $K_{(2)}$ and $K_{(4)}$, calculated from the slopes and the values of k_d and k_2' from Table I, are presented in Table II. Table II also includes dimerization constants determined photometrically in aqueous solution.

The constants for dimerization of ^+HDOH and DOH showed fairly good agreement, indicating that interaction between the molecules is due to their ring systems (26). This result is further supported by the fact that no dimerization of the phenolate form (DO^-) was found; *i.e.*, dimerization was prevented by the negative charge in the ring system.

The constants for dimerization of I given in Table II differ considerably from those presented elsewhere (15), which were determined at such high concentration ranges that I probably was mainly present as higher aggregates.

A test of an extraction model including formation of the dimer and trimer gave about the same residual variance as the model including formation of the dimer and tetramer but was unreliable because of the fact that the estimates $a_1 < 0$ and $a_0 > 1/D_{\min}$ (25).

The constants for dimerization and tetramerization of I and II presented are of such a high magnitude that they have to be taken into account not only for calculations of distribution ratios at $C_D > 10^{-6} M$ but also for the determination of binding constants, *e.g.*, in DNA complex (27–29).

Disturbing association processes are probably responsible for the great difference between published data on acid dissociation constants of the amino group ($pK = 8.2$ – 8.99) determined by potentiometric titration of approximately $10^{-3} M$ solutions (30–32) and those presented in this paper.

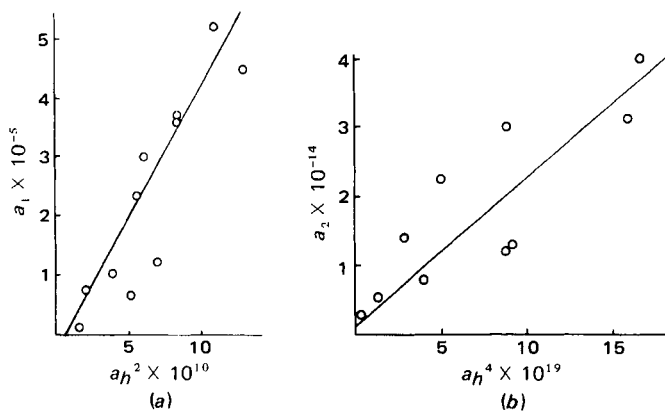


Figure 6—Evaluation of constants for dimerization (a) and tetramerization (b) of I. (Conditions were as given in Table II.)

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