THE HYDROLYSIS ACTIVATION OF THE DOXORUBICIN CARDIOPROTECTIVE AGENT ICRF-187 ((+)-1,2-BIS(3,5-DIOXOPIPERAZINYL-1-YL)PROPANE)

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ABSTRACT:

ICRF-187 ((+)-1,2-bis(3,5-dioxopiperazinyl-1-yl)propane) has shown promise [Speyer et al., N. Engl. J. Med. 319, 745 (1988)] as a cardioprotective agent against what may be an iron-based doxorubicin (Adriamycin)-induced cardiotoxicity. ICRF-187 likely exerts its action through its rings-opened hydrolysis product, a compound that has an EDTA-type structure and, likewise, strongly binds metal ions. The hydrolysis of ICRF-187 was followed spectrophotometrically in the ultraviolet and was shown to be pseudo-first-order over a wide pH range. The hydrolysis mechanism was shown to have a hydroxide-catalyzed path and a pH-independent path similar to the hydrolysis of other imides. The anionic form of ICRF-187 (with a pKₐ of 9.6 at 37°C) was resistant to hydroxide attack. The kinetically, spectroscopically and potentiometrically determined pKₐ values were all in excellent agreement and thus provided a test of the mechanism. Each inside group underwent hydrolysis and ionization independently of the other. The rate of ICRF-187 hydrolysis was also followed by observation of the removal of Cu²⁺ from a Cu²⁺-doxorubicin complex by the ICRF-187 hydrolysis product.

The clinical use of the antitumor drug doxorubicin (Adriamycin) is limited by a unique, potentially fatal cumulative dose-dependent cardiotoxicity (1). Iron-dependent Fenton-type free radical oxidative stress on the relatively unprotected cardiac muscle (1,2) may be responsible for doxorubicin’s cardiotoxicity. Lipid peroxidation and damage to other cellular components induced by doxorubicin has been shown to depend upon the presence of iron (1, 3–9) or copper (1, 6). ICRF-187 (ADR-529) is the most promising of the cardioprotective agents (10–16) that have been used in attempts to reduce doxorubicin cardiotoxicity (10–16). In randomized clinical trials (12, 13, 15, 16) ICRF-187 has provided significant reductions in doxorubicin cardiotoxicity. ICRF-187 is the water-soluble (S)(+)-enantiomer of the racemic compound ICRF-159 (Razozone). Upon full hydrolysis, this bis-cyclic imide derivative gives an optical isomer of ICRF-198, which has a structure similar to EDTA, and is likewise a powerful metal ion chelating agent (17, 18). ICRF-187 and doxorubicin have been shown to interact synergistically in vitro to enhance S-180 cell kill (19). Although ICRF-159 has been shown to diffuse passively into cultured cells (18), its charged hydrolysis product, ICRF-198, is itself presumably too polar to cross cell membranes. The uptake and subsequent hydrolysis of ICRF-187 in the myocardium may thus protect cellular components by reducing the amount of free iron and copper available for formation of oxygen radical-forming metal ion-doxorubicin complexes. This study was undertaken to determine the mechanism of the activation of ICRF-187 to its metal-ion binding hydrolysis product. Knowledge of the rate of hydrolysis may be useful for predicting the appearance of the pharmacological effects of ICRF-187. Knowledge of the mechanism of hydrolysis may also allow a prediction of structure-activity effects and new insights for synthesizing related compounds with differing rates of hydrolysis.

Materials and Methods

Materials. Doxorubicin was obtained from Adria Laboratories (Columbus, OH) and its concentration was determined spectrophotometrically in methanol by use of ε 13,050 M⁻¹ cm⁻¹ at 477 nm (20). ICRF-187 (ADR-529), as the free base (purity 95.9% by HPLC) and ICRF-198, were also obtained from Adria Laboratories and used as received. Stock solutions (1 mg/ml) were prepared in water and either used immediately or frozen in small aliquots for later use. Buffers and CuSO₄ were of the highest grades available. Unless otherwise noted, Tris buffers contained 50 mM Tris and 150 mM KCl, and ammonia buffers contained 50 mM NH₄Cl and 150 mM KCl. The pH 7.0 phosphate buffer was prepared from Na₂HPO₄ (Fisher) and Na₂HPO₄ (BDH) and had a final phosphate concentration of 66 mM.

Spectrophotometric Measurements. Spectra and absorbance-time traces of solutions were recorded in stopped silicone 1-cm cells on a Shimadzu UV-260 recording spectrophotometer equipped with a thermostatted cell holder. The Cu²⁺-doxorubicin complex (21), used for the determination of the metal ion-binding product of ICRF-187 hydrolysis, was formed directly in the spectrophotometer cell by adding microliter amounts of CuSO₄ to doxorubicin in buffer.

Results

Spectrophotometric Determination of the pKₐ of ICRF-187. In neutral pH buffers ICRF-187 displays a peak that appears at 205–211 nm. In more basic solutions this peak is replaced by a peak with a maximum at 227 nm (fig. 1), which is due to the ionization of the relatively acidic imide group to its corresponding anionic form. Since the absorbance in alkaline solutions decreases with time as hydrolysis proceeds, the absorbance at zero time was estimated by extrapolating the absorbance-time traces back to time zero. Plots of the absorbance, A, at 227 nm as a function of pH (fig. 2) were best fit by three-parameter nonlinear least squares analysis to an equation for a species with a single pKₐ.

\[ A = (A_1 + A_2K_a[H^+]/(1 + K_a/[H^+])) \] (1)
where $A_1$ and $A_2$ are the absorbances at 227 nm of the imide and imide anion forms of ICRF-187. At 25°C the best fit values yield the corresponding $\epsilon_1$ and $\epsilon_2$ of $1100 \pm 300 \text{ M}^{-1} \text{ cm}^{-1}$ and $37,400 \pm 400 \text{ M}^{-1} \text{ cm}^{-1}$, respectively. The errors quoted are fitting errors only. The $\epsilon$'s were calculated from $A_1$ and $A_2$. The concentration of ICRF-187 was computed from the HPLC determination of purity. The calculated $pK_a$ values and the $\Delta H$ values derived from them are given in Table 1. Other models with 2 $pK_a$ values were tried in an attempt to find a better fit for the data. They were all rejected because they offered no improvement in the overall fit (standard error of fit 0.0153 $A$ units for 2 $pK_a$ model over the 1 $pK_a$ model (standard error of fit of 0.0145 $A$ unit). In the 2 $pK_a$ model, the error on the second $pK_a$ was large ($\pm 2.0$ $pK_a$ units), which indicated that this parameter was not well defined.

There are two possibilities to explain why the absorbance data was best fit to eq. 1 for a single $pK_a$. The first is that the dissociation of the second proton occurs at a $pH$ much higher than the $pK_a$ determined in eq. 1. The second is that each proton dissociates with the same $pK_a$. In order to rule out the first of these two possibilities, spectra were run in 0.10 M NaOH/150 mM KCl. The spectrum was unchanged from that seen at $pH$ 11.4 thus indicating that no new deprotonated species were formed under highly basic conditions. The absorbance at 227 nm (extrapolated back to zero time) was also measured in basic

![Graph of absorbance vs. pH](image)

**FIG. 1.** Top: $A$, $A'$, $A''$, etc., are spectra of 30 $\mu$M ICRF-187 at 25°C in pH 10.8 ammonia buffer recorded at intervals of 5.0 min. B is the spectrum of 30 $\mu$M ICRF-198 in the same ammonia buffer. C is the spectrum of 30 $\mu$M ICRF-187 in pH 7.4 Tris buffer. D is the spectrum of 30 $\mu$M ICRF-198 in the same Tris buffer. Bottom: Spectrum of 50 $\mu$M ICRF-187 at 37°C in pH 9.31 ammonia buffer recorded at intervals of 5 min. The scan was started at 300 nm, about 20 s after the start of mixing. The absorbance decreased with time.

**TABLE 1**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Temperature</th>
<th>$\Delta H$ or $\Delta H^°$ (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{OH}$ (M$^{-1}$ min$^{-1}$)</td>
<td>230 ± 10</td>
<td>820 ± 50</td>
</tr>
<tr>
<td>$k_a$ (min$^{-1}$)</td>
<td>(1.0 ± 0.1) $\times$ 10$^4$</td>
<td>(2.2 ± 1.1) $\times$ 10$^4$</td>
</tr>
<tr>
<td>$pK_a$</td>
<td>10.00 ± 0.02</td>
<td>9.45 ± 0.05</td>
</tr>
<tr>
<td>$pK_a$'</td>
<td>10.03 ± 0.02</td>
<td>9.63 ± 0.01</td>
</tr>
<tr>
<td>$k_{aOH}$ (min$^{-1}$)</td>
<td>1.6 $\times$ 10$^4$</td>
<td>7.1 $\times$ 10$^4$</td>
</tr>
<tr>
<td>$t_{1/2}$ (hr)</td>
<td>73</td>
<td>16.3</td>
</tr>
<tr>
<td>$t_{90}$ (hr)</td>
<td>36</td>
<td>8.2</td>
</tr>
</tbody>
</table>

* In buffers containing 150 mM KCl.
* Kinetically determined from the fit of eq. 3 to the data of fig. 4.
* Spectrophotometrically determined at 227 nm from the fit of eq. 1 to the data of fig. 2.
* Potentiometrically determined.
* At pH 7.40.
* The half-time for the decrease in the total absorbance change at pH 7.40.

The solid lines are best fits for single ionizations with $pK_a$ values of 10.03 ± 0.02 at 25°C and 9.63 ± 0.01 at 37°C. At 25°C and 37°C ammonia buffers were used at all pH values higher than 9.26 and 8.83, respectively; all others were Tris buffers.

The solutions containing NaOH and 150 mM KCl. If a separate deprotonation of the second ring did occur in these very basic solutions, it would be expected to produce either a new peak or an absorbance at 227 nm that was approximately twice $A_2$; but this was not seen. Thus deprotonation of both rings occurs simultaneously with the same $pK_a$ values. Apparent $\epsilon$'s of: 34,700 M$^{-1}$ cm$^{-1}$; 35,600 M$^{-1}$ cm$^{-1}$ and 35,900 M$^{-1}$ cm$^{-1}$ were measured in 0.010, 0.10 and 0.30 M NaOH/150 mM KCl, respectively. These values closely approach the value of 37,400 ± 400 M$^{-1}$ cm$^{-1}$ determined from eq. 1 at much lower pH values, thus suggesting that the $pK_a$ measured is for formation of the fully deprotonated dianion. Thus each imide proton is dissociating
from ICRF-187 with the same $pK_a$. This is as might be expected considering the distance the two rings are apart.

**Potentiometric Titration of ICRF-187.** Titration of 1.0 mM ICRF-187 by 0.10 M NaOH and 0.10 M HCl (on the basic and acidic legs of the titration, respectively) in 150 mM KCl was followed on a pH meter. Assuming that the two imide groups ionize with the same $pK_a$ values, the titration $pK_a$ values (which are equal to the microscopic $pK_a$ values), as determined from KCl blank-corrected pH titration curves (plots not shown) were 10.1 (for both imide groups), 2.5 and <2 at 25°C; and 9.4 (for both imide groups) at 37°C (acid leg not determined). These $pK_a$ values are less accurate than the spectrophotometric and kinetically determined $pK_a$ values (Table 1) due to hydrolysis of the imide group that occurs during the course of the titration. Nonetheless, they are in good agreement with the other determinations and these results do confirm that the two imide groups ionize with the same $pK_a$. As a comparison, the $pK_a$ values for ICRF-198 are 7.55, 3.80 and 1.72 (37°C) (17).

**Kinetics of the Hydrolysis of ICRF-187 and the Kinetic Determination of Its $pK_a$.** The changes in absorbances with time, such as those shown in Fig. 1, at a variety of different wavelengths were best fit to an equation for a three term single exponential equation of the form:

$$ A = \Delta A (1 - e^{-k_{obs}t}) + A_0 $$

where $\Delta A$ is the final minus the initial absorbance, $A_0$ is the initial absorbance and $k_{obs}$ is the observed pseudo-first-order rate constant (eq. 3).

There are at least two reaction mechanisms that can produce a single exponential decay of the absorbance. The first and simplest is scheme A shown in fig. 5, which has a single rate-determining step for the opening of the first ring followed by a much faster opening of the second ring. (In order to simplify presentation of this mechanism, scheme A is shown with the ionization occurring only on the right-hand ring). The dependence of $k_{obs}$ on pH (fig. 4) is consistent with reaction scheme A shown in fig. 5, in which there are two pathways for hydrolysis; a base-catalyzed and a pH-dependent pathway. This latter pathway might be either a unimolecular decomposition or a reaction with water. In scheme A the anionic form is essentially resistant to OH$^-$ attack, presumably because of the negative charge on the molecule. The mechanism shown in fig. 5 predicts that

$$ k_{obs} = \frac{k_a + k_{OH} [OH^-]}{1 + K_a/[H^+]}, $$

where $k_{OH}$ is the second order rate constant for the reaction of OH$^-$. $k_a$ is a first order rate constant for a pH-independent unimolecular decomposition (or reaction with water) and $K_a$ is

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**Fig. 4. Plots of $k_{obs}$ vs. pH for the hydrolysis of ICRF-187 at 25°C (O) and 37°C (●).**

The data through which the broken line (---) falls to the scale on the left and for the continuous line (-----) that on the right. The curved lines are best fits for a mechanism with a single $pK_a$ as described in the text. The buffers used were those described in fig. 2.

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**Scheme A**

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**Scheme B**

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**Fig. 5. Top: Reaction Scheme A for the hydrolysis of ICRF-187. Product D is actually an optical isomer of ICRF-198. Bottom: Reaction Scheme B for the hydrolysis of ICRF-187.**

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The reactants A, B and D are the species labeled as such directly above. Species C is the other monocyclic imide but with the left-hand ring of ICRF-187 opened instead of the right. Not shown for clarity are the imide ionizations (2 on species A, 1 on each of B and C), each of which are assumed to have the same $pK_a$ values. Additionally, $k_{obs}$ is a function of $k_{OH}$, $k_a$ and $K_a$ as given by eq. 3.
an acid dissociation constant of ICRF-187. The [OH\(^{-}\)] was calculated from \([\text{OH}^{-}] = K_w/[\text{H}^{+}]\), where \(K_w\) is the ion product of water.

A second more complex mechanism, scheme B, shown also in fig. 5, also predicts a single exponential decrease in absorbance, provided that certain criteria are met. These are: (a) the rate constants \((k_{obs})\) for ring opening are the same for all species and are given by eq. 3; (b) the \(pK_a\) values for the imide ionization of all species are the same; (c) the molar absorption coefficient of each closed ring is the same; and (d) the molar absorption coefficient of each open ring is the same. Thus this mechanism assumes that hydrolysis and deprotonation of each ring occurs independently of the other. Again, if the distance between the rings is considered, this is a reasonable assumption. A solution to the differential equations for scheme B gives an equation that is of a form identical to eq. 2. Because each ring undergoes hydrolysis independently of the other, the pH dependence of \(k_{obs}\) for scheme B is also given by eq. 3. As species \(A\) may hydrolyze by either of two equivalent steps (to \(B\) or to \(C\) with the same \(k_{obs}\)) the concentration of \(A\) (ICRF-187) is given by:

\[
[A] = [A]_0 e^{-2k_{obs}t}
\]  

(4)

where \([A]_0\) is the initial concentration of \(A\). Thus, while the absorbance decays with a rate constant of \(k_{obs}\) (eq. 2), because both imide groups are equivalent and independent, the concentration of \(A\) (ICRF-187) decays with an apparent rate constant of 2 \(k_{obs}\).

More complex mechanisms, to explain the pH dependence of \(k_{obs}\), including either acid-catalyzed terms, or base-catalyzed terms for reaction of the anionic form did not result in any significant improvements to the fit. The best fit parameters obtained by nonlinear least squares analysis of the data in eq. 3 are given in table 1. The values of \(\Delta H^\circ\) were calculated from \(\Delta H^\circ = E_a - 0.6\) kcal where \(E_a\) is the Arrhenius activation energy. At 37°C the water term \((k_w)\) is less well defined than is the hydroxide term \((k_{obs})\) due to its smaller contribution to the overall rate. It can be estimated from the parameters of table 1 that at pH 7.4 and 37°C the hydroxide-catalyzed path accounts for 70% of the observed rate. The value of \(k_{obs}\) was also measured at pH 2.93 and found to have a value of \(6 \times 10^{-3}\) min\(^{-1}\) which is more than ten-fold slower than that observed at pH 7.4. This result might be taken as additional evidence for the existence of a water term, as was found by Zerner and Bender (22) for phthalimide hydrolysis or possibly a new contribution from an acid-catalyzed hydrolysis term (22). A good test of the mechanism (scheme B) shown in fig. 5 is the excellent agreement between the spectrophotometrically determined \(pK_a\) values and the kinetically determined \(pK_a\) values. A lack of agreement between the \(pK_a\) values would have necessitated more complex mechanisms than those shown in fig. 5.

The kinetics of the hydrolysis of ICRF-187 followed by its reaction with the Cu\(^{2+}\)-doxorubicin complex. Cu\(^{2+}\)-doxorubicin forms a 2 drug to 1 Cu\(^{2+}\) complex that has been characterized spectrophotometrically (21). The decrease in absorbance at 600 nm that occurs when a chelating agent such as ICRF-198 (17) that binds Cu\(^{2+}\) more strongly than doxoorubicin (1, 21) (and is thus capable of removing Cu\(^{2+}\) from the Cu\(^{2+}\)-doxorubicin complex) was also used to follow the time course of hydrolysis of ICRF-187 (fig. 6). The data of fig. 6 could not be adequately fitted by a single exponential as expected for reaction scheme B.

This might occur because the single ring-opened species (B and C) may also remove Cu\(^{2+}\) from Cu\(^{2+}\)-adriamycin, thus complicating the kinetic analysis. However, assuming 1 mol of hydrolysis product reacts with 1 mol Cu\(^{2+}\), it can be calculated from the initial velocity by use of \(k_{obs} = v/[ICRF-187]_0\), that \(k_{obs}\) is \((1.1 \pm 0.1) \times 10^{-4}\) min\(^{-1}\) at 25°C. This compares to the value determined spectrophotometrically in the UV (table 1). Similarly, an average \(k_{obs}\) of \(3.5 \times 10^{-4}\) min\(^{-1}\) (plots not shown) was also determined at 37°C in pH 7.0 Tris buffer and can be compared to the UV-determined \(k_{obs}\) of \(3.8 \times 10^{-4}\) min\(^{-1}\) (fig. 4) under identical conditions. The value of \(k_{obs}\) \((4.1 \times 10^{-4}\) min\(^{-1}\) in 66 mM sodium phosphate buffer under the same conditions was virtually unchanged which rules out a significant buffer-catalyzed hydrolysis pathway in the mechanism. Also the fact that upon changing from the Tris buffers to the ammonia buffers did not result in any significant discontinuity in the \(k_{obs}\) plots of fig. 4 weighs against any significant ammonia catalysis term.

Discussion

Dawson (18) in a study with \(^{14}C\)-labeled ICRF-159 showed that the hydrolysis of ICRF-159 was faster at higher pH and decreased exponentially with a half-time of 7.6 hr (37°C, pH 7.4 phosphate buffer) and an apparent first order rate constant of \(1.53 \times 10^{-3}\) min\(^{-1}\). Since Dawson was measuring the disappearance of ICRF-159 only, corresponding to species A in fig. 5, its concentration is governed by eq. 4. The apparent rate constant for this process is \(k_{obs}\) (eq. 4), which is twice the rate constant determined from the change in absorbance with time, \(k_{obs}\) (eq. 2). Thus using Dawson's data it is possible to calculate \(k_{obs}\) for the opening of the single ring of \(k_{obs} = 1.53 \times 10^{-3}\) min\(^{-1}\) /2 = 7.6 \times 10^{-4}\) min\(^{-1}\). This is in good agreement with the \(k_{obs}\) determined spectrophotometrically of \(7.1 \times 10^{-4}\) min\(^{-1}\) (table 1). This agree-
ment is also evidence for scheme B, as scheme A gives a half-
time for the reaction that is too long by a factor of 2. Further
evidence for scheme B comes from the observation by Dawson
(18) that TLC of ICRF-159 incubated at pH 7.4 and 37°C showed
three breakdown products. The presence of intermediates also
strongly favors scheme B over scheme A, in which no interme-
diates should be detectable. Additionally, the fact that deproton-
ation of both rings occur with the same pKₐ, which is a necessary
condition for scheme B, but not for scheme A, means scheme B
cannot be ruled out. The fact that the Cu⁺⁺doxorubicin assay,
which presumably measures only both-rings-opened product D,
could not be fit to a single exponential is further evidence in
favor of scheme B over scheme A, which would predict a single
exponential increase in D with time while scheme B does not.
Dawson also showed that ICRF-159 passively diffuses through
the cell membrane and hydrolyzes at the same rate in the
presence of cultured cells in tissue culture medium, indicating a
lack of any other significant pathway for hydrolysis.

The hydrolysis of ICRF-187 in phosphate buffered saline (pH
7.4, 37°C) has also been followed by HPLC and mass spectrom-
etry (23). These results were also consistent with scheme B,
inaasmuch as three hydrolysis products were identified, one
of which was ICRF-198 and the other two being the monoacid
monoamide single ring-opened intermediates (species B and C
of fig. 5). The ICRF-187 itself was observed to undergo 47%
hydrolysis in 21 hr. A biological half-life of 12 hr for ICRF-159
has also been measured at 37°C and pH 6.8–7.0 (24).

Mechanistic studies on the hydrolysis of imides (22, 25–27)
have shown that the pH dependence of the reaction is due to a
OH⁻-catalyzed step (25), similar to peptide bond hydrolysis.
While most studies have been carried out at alkaline pH (25–
27), studies at neutral pH measured a kₜ for 5.4 × 10⁻⁹ min⁻¹
at 100°C for phthalimide (22) and 1.1 × 10⁻² min⁻¹ at 80°C for
ethosuximide (26), which can be compared to a value of 2.2 ×
10⁻⁴ min⁻¹ at the lower temperature of this study, 37°C. The
value of kₜ for the base-catalyzed path of ICRF-187 hydrolysis
of 230 M⁻¹ min⁻¹ can be compared to a kₜ for 204 M⁻¹ min⁻¹
and 45 M⁻¹ for succinimide and diacetylamino, respectively,
at 25°C (25) and 62 M⁻¹ min⁻¹ for ethosuximide at 80°C (26).
Ethosuximide was found to have a ΔH of 22 kcal/mol (26)
under conditions where base-catalyzed ICRF-187 hydrolysis
predominates, which is similar to the value of 18 kcal/mol found
for base-catalyzed ICRF-187 hydrolysis (table 1). Thus compar-
ison of ICRF-187 hydrolysis with hydrolysis of other imides
indicate that ICRF-187 hydrolysis occurs by a similar mechanism
and at similar rates. A study (27) on the hydrolysis of substitut-
ated phthalimides at 25°C showed that kₜ varied from 220 M⁻¹
min⁻¹ (–NH₂) to 10,500 M⁻¹ min⁻¹ (–NO₂). The fact that imides
of differing structure (22, 25–27) hydrolyze at significantly dif-
fering rates suggest that it may be possible to synthesize ICRF-
187 derivatives of related structure that hydrolyze either faster
or slower than ICRF-187. It might, for example, be desirable to
synthesize faster hydrolyzing, faster acting ICRF-187 derivatives,
thus possibly improving its pharmacological properties. The pKₐ
of 10.03 for ICRF-187 at 25°C can be compared to a pKₐ of 9.6
for succinimide (25) under similar conditions and pKₐ values
of 9.06 for ethosuximide (26) and 11.63 for glutarimide (25).

The hydrolysis of ICRF-187 is also promoted by Cu⁺⁺ (17) and
an Fe⁺⁺ complex of doxorubicin (28, 29). In pH 7.4 Tris buffer
the Fe⁺⁺doxorubicin complex reacts with ICRF-187 with an
apparent second order rate constant, k, of 123 M⁻¹ min⁻¹.
Equating the initial rate at which this reaction takes place with
the initial rate at which ICRF-187 undergoes hydrolysis, under
the same conditions (table 1) and with

\[-d[ICRF-187]/dt = k[Fe^{3+}-doxorubicin][ICRF-187] = k_{obs}[ICRF-187]

allows an estimate of the Fe⁺⁺doxorubicin concentration (1.7
μM Fe⁺⁺) at which the two rates become equal. If even this low
concentration of Fe⁺⁺doxorubicin were to be present in vivo
then the metal ion complex-promoted pathway could also be
significant in the hydrolysis activation of ICRF-187.

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